Characterisation of Prostaglandin E receptors

by

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Prostaglandin (PG) E_2 has been shown to act on at least three distinct receptor subtypes, designated EP_1, EP_2 and EP_3, with recent evidence suggesting the possibility of a fourth EP-receptor subtype. Whilst good antagonists are not available, a range of PGE analogues of differing selectivity for the three receptor subtypes may be used to determine which is effective in a given system. The activity of these analogues has not yet been assessed on the putative EP_4-receptor containing preparation. This research has involved the use of such compounds in an *in vivo* model of rabbit skin inflammation, and biochemical studies on both platelets and cultured macrophages, to determine the subtype(s) of PGE receptor mediating the pro-inflammatory, pro-aggregatory, and anti-inflammatory effects, respectively, of PGE_2.

Whilst PGE_2 alone had little effect on rabbit skin inflammation, potentiation of vascular permeability induced by mediators of inflammation, such as bradykinin (BK) and FMLP was observed. The potentiation has been attributed to the vasodilator activity of PGE_2 which is typically mediated via the EP_2-receptor subtype. However the finding that compounds with both EP_2- and EP_3-receptor activity were the most active, suggested that vasodilatation was not the sole mechanism of the potentiation. A comparison of the ability of PGE_2 and the stable PGI_2 analogue, cicaprost, to induce vasodilatation and potentiate the responses to both BK and FMLP, was consistent with this suggestion. It has since been proposed that there is an initial EP_3-mediated, dilatation-independent component to the potentiation of BK by PGE_2.

PGE_2 was shown to potentiate aggregation in human platelets possibly through inhibition of adenylate cyclase. The potencies of seven PGE analogues as potentiators of the PAF-induced aggregation were assessed, and the ranking indicated that the EP_3-receptor subtype may be involved. This was supported by studies on the adenylate cyclase second messenger system. The ability of a series of PGE analogues to inhibit the cicaprost-induced rise in cyclic AMP in human washed platelets was measured. The EP_3-selective agonists potently inhibited cyclic AMP production, in

comparison to the weak effect of PGE analogues selective for the EP_1- and EP_2- receptor subtypes.

Evidence suggests PGE_2 has a major role in the modulation of interleukin (IL) -2 production by lymphocytes. The release of IL-1 from accessory macrophages, besides stimulating release of IL-2 from lymphocytes, also stimulates the release of PGE_2 from other macrophages. PGE_2 acts as a negative feedback, inhibiting production of IL-1 by the macrophages, and consequently IL-2 by the lymphocytes. Human promyelocytic leukaemia (HL-60) cells grown in suspension culture differentiate into macrophage-like cells when exposed to a phorbol diester. Plasma membranes were prepared from differentiated HL-60 cells by homogenisation and sucrose density centrifugation. Of the four EP_2-receptor agonists tested, only 11-deoxy PGE_1 displaced binding in a manner comparable to PGE_2 itself. The lack of activity of EP_1- and EP_3-selective compounds suggests that the receptor is not of either subtype. By implication this may represent the fourth EP-receptor subtype.

I declare that this thesis has been composed by myself and the research has been conducted solely by me.

The results of the rabbit model of skin inflammation and inhibition of cyclic AMP production in human platelets are discussed in the context of contemporaneous experiments conducted by my colleagues, as clearly referred to in the text.

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Publications

Abstracts

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ABBREVIATIONS

arachidonic acid	AA
bradykinin	BK
diacylglycerol	DAG
dihomo-γ-linolenic acid	DGLA
endothelium-derived relaxing factor	EDRF
equipotent molar ratio	e.m.r.
essential fatty acid	EFA
f-met-leu-phe	FMLP
hydroxyeicosatetraenoic acid	HETE
5-hydroxytryptamine	5-HT
inositol 1,4,5-trisphosphate	IP_3
interleukin	IL
leukotriene	LT
lipopolysaccharide	LPS
lipoxin	LX
non-steroidal anti-inflammatory drug	NSAID
1-oleoyl-2-acetyl-glycerol	OAG
phosphatidic acid	PA
phosphatidylinositol	PI
phosphodiesterase	PDE
phospholipase	PL
pituitary adenylate cyclase activating polypeptide	PCAP
plasma activation permeability agent	PAPA
platelet activating factor	PAF
platelet-derived growth factor	PDGF
polymorphonuclear	PMN
prostaglandin	PG
protein kinase	PK
12-o-tetradecanoyl phorbol 13-acetate	TPA
thromboxane	TX

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Chapter 1

General Introduction

1.1 Prostanoid biosynthesis

Prostanoids, that is prostaglandins (PGs) and thromboxanes (TXs), may be synthesised *de novo* in almost every cell in the body, with the exception being red blood cells. They are released in response to stimuli, including hormones, chemical mediators, mechanical stimuli and cell injury. The PGs and TXs are formed from the 20-carbon-containing fatty acid constituents of membrane phospholipids, dihomo-γ-linolenic acid, arachidonic acid and eicosapentaenoic acid. These fatty acids give rise to the 1-, 2- and 3- series prostanoids respectively (Bergstrom *et al.*, 1964,1968; Van Dorp, 1964; Samuelsson *et al.*, 1966,1978; Bergstrom 1967), each referring to the number of double bonds in the side chains. Since arachidonic acid is the predominant polyunsaturated fatty acid present in mammalian cells, the 2-series prostanoids are the most abundantly formed of the three (Moncada *et al.*, 1980).

Prostanoids are formed from arachidonic acid by the cyclooxygenase pathway (see Needleman *et al.*, 1986; see Williams & Higgs, 1988) as illustrated in Figure 1.1. Leukotrienes (LTs), lipoxins (LXs) and hydroxyeicosatetraenoic acids (HETEs) are also formed from arachidonic acid, and together with the prostanoids constitute the eicosanoids (see Needleman *et al.*, 1986). The lipoxygenase enzymes catalysing their formation do not appear to be as widely distributed as those for the prostanoids, but have been recognised in platelets, neutrophils and the skin.

Before enzymatic attack can occur, arachidonic acid must first be released from the membrane phospholipid in which it is incorporated in an esterified form (Billis *et al.*, 1975); three pathways have been proposed. Cleavage from the 2-position of the phospholipid is catalysed by the action of phospholipase (PL) A_2 (Flower & Blackwell, 1976; Vogt, 1978). Whilst PLA_2 has been reported to play the major role in the activation of platelets (Broekman, 1986; Derksen & Cohen, 1975;

Mahadevappa & Holub, 1986; Purdon *et al.*, 1987), others believe there is insufficient PLA_2 activity to account for the burst of arachidonate that occurs after platelet stimulation (Bell *et al.*, 1979). These groups did however detect sufficient diglyceride lipase activity and suggested that in the platelet the PLC pathway is responsible for the release of arachidonic acid, forming initially diacylglycerol (DAG) from which arachidonic acid is released by the action of this diglyceride lipase. Another study (Billah *et al.*, 1980) suggests the involvement of both PLC and PLA_2 in sequence, with PLC producing phosphatidic acid (PA) from phosphatidylinositol (PI), subsequent PLA_2 activity releasing arachidonic acid.

Irrespective of the phospholipase involved, free arachidonic acid is sequentially converted to the PG endoperoxides, PGG_2 and PGH_2, by PG endoperoxide synthetase (see Needleman *et al.*, 1986), and then to tissue specific PGs. Consequently, TXA_2 is formed in platelets (Needleman *et al.*, 1976) and PGI_2 in endothelial cells (Moncada *et al.*, 1976). Arachidonic acid not converted into the eicosanoids is reesterified into phospholipid, thereby restoring the ability to release the PG precursor upon subsequent stimulation.

It is proposed that liberation of arachidonate is mediated by at least two distinct PLA_2s in mammalian cells (Takayama *et al.*, 1991; Fujimori *et al.*, 1992). The 14 kDa form may be subdivided into group I, in mammalian pancreas, and group II, in inflammatory exudate, according to its primary structure. The requirement of mM Ca^{2+} in order to function suggests the 14 kDa form exerts its action extracellularly. Here it may have a role in cell membrane homeostasis and digestion. Recently, the secretory PLA_2 of the group II type was isolated from rheumatoid arthritic synovial fluid, and the gene encoding it cloned (Seilhamer *et al.*, 1989).

A 90 kDa form has also now been isolated and is believed to function intracellularly. This novel cytosolic PLA_2 has also recently been cloned and expressed from the human monoblast cell line, U937 (Clark *et al.*, 1991). It is known to bind to membranes in a Ca^{2+}-dependent manner (Clark *et al.*, 1991), and phosphorylation

has been reported to have an important role in its agonist-induced activation (Lin *et al.*, 1992). There is no reported structural similarity with the well-known mammalian secretory PLA_2.

PG endoperoxide synthetase, catalysing the subsequent formation of PGG_2 and PGH_2, consists of the two enzymes cyclooxygenase and hydroperoxidase combined in a single protein unit. Immunocytochemistry has shown cyclooxygenase to be located on the endoplasmic and nuclear membranes in Swiss mouse 3T3 fibroblasts, but not on the mitochondrial or plasma membranes (Rollins & Smith, 1980). Recent evidence suggests that lipid bodies may represent additional sites of PG endoperoxide synthetase localisation in murine fibroblasts and both human eosinophils and monocytes (Weller & Dvorak, 1992).

A clone encoding PG endoperoxide synthetase activity has been isolated from sheep vesicular gland (Merlie *et al.*, 1988; Yokoyama *et al.*, 1988), mouse 3T3 fibroblasts (DeWitt *et al.*, 1990) and the human genomic library (Yokoyama & Tanabe, 1989). The amino acid sequences encoded by the mouse and human cDNAs demonstrated 88 % and 91 % homology with the sheep enzyme respectively.

In addition, an mRNA for PGG / H synthetase has been isolated from sheep tracheal mucosal epithelial cells (Rosen *et al.*, 1989). It is suggested that this mRNA may demonstrate high homology to the sarcoma virus - induced mRNA recently isolated from chick embryo fibroblasts by Xie *et al.* (1991), though further investigation following cloning and amino acid sequencing of the former is necessary to verify this. In non-proliferating cells the chick mRNA was at a low level and was nonfunctional. The protein it expressed in proliferating cells, demonstrated 59 % homology with the sheep PGG / H synthetase already cloned. Comparison of their respective mRNAs suggests this may be a new inducible form of the enzyme. Indeed, induction of a novel cyclooxygenase has recently been reported to be responsible for endotoxin priming of rabbit alveolar macrophages for amplified synthesis of prostanoids (O'Sullivan *et al.*, 1992).

A second form of cyclooxygenase, the PGG_2 synthetase component of PG endoperoxide synthetase, is also reported to have been isolated from human umbilical vein endothelial cells, with 61 % homology to the previously isolated cyclooxygenase polypeptide (Hla & Neilson, 1992). Messenger RNA for both cyclooxygenases was also expressed in vascular smooth muscle cells, monocytes and fibroblasts, and was differentially regulated. In addition, two pools of cyclooxygenase have been found to exist in murine fibroblasts, with 63 % homology between the corresponding proteins (Winn *et al.*, 1992). The first is a constitutive, long-lived cyclooxygenase, the second being regulated by corticosteroids (see page 9) and rapidly turned over.

This cyclooxygenase component catalyses the insertion of two molecules of oxygen into arachidonic acid, giving rise to the 15-hydroperoxy compound, PGG_2. The hydroperoxy group is subsequently reduced to hydroxy, by the action of hydroperoxidase. The resulting PGH_2 then serves as the precursor for the PGs and TXs, the conversion dependent on the specific enzyme(s) present in a particular tissue. The PG endoperoxides, PGG_2 and PGH_2, are unstable and will decompose with a half-life ($t_{1/2}$) at 37 °C of ~ 4-5 min, in the absence of these enzymes.

Since there are efficient mechanisms for the inactivation of the prostanoids, their action is normally of short duration. Initially the prostanoids must be transported across the membrane back into the cells where the catabolic enzymes are. The breakdown process itself occurs in two stages. The first is a rapid prostanoid-specific breakdown involving dehydrogenation of the carbon-15 hydroxyl group by the widely distributed 15-hydroxy prostaglandin dehydrogenase, producing 15-keto compounds (reviewed in Hansen, 1976). These products then enter a slower oxidation phase. PGI_2, $t_{1/2}$ at 37 °C of ~ 3 min (Dusting *et al.*, 1977), and TXA_2, $t_{1/2}$ at 37 °C of ~ 30 s (Hamberg *et al.*, 1975), are themselves chemically labile and rapidly decompose to the essentially biologically inactive intermediates 6-keto-$PGF_{1\alpha}$ and TXB_2 respectively, before being metabolised further. The levels of these metabolic products in biological fluids are usually taken to be

representative of the rates of production of the individual prostanoids, but this is complicated by the fact that there may be many degradation products.

Besides the cyclooxygenase-derived PGs, the existence of a series of $PGF_{2\alpha}$-like compounds which are produced *in vivo* in humans by a non-cyclooxygenase, free radical - catalysed mechanism has recently come to the fore (Morrow *et al.*, 1990). Reactive free radicals may be formed pathophysiologically in disorders such as atherosclerosis, ischaemia-reperfusion injury, inflammatory disease, cancer and ageing. The finding that one of these compounds, namely 8-epi-$PGF_{2\alpha}$, has biological activity, suggests they may in fact be mediators in such conditions.

8-Epi-$PGF_{2\alpha}$ was indeed found to reduce renal blood flow and the glomerular filtration rate when infused into the rat kidney (Morrow *et al.*, 1990). This effect is reported to be mediated through stimulation of PI turnover, via interaction with the TXA_2 / PGH_2 receptor (Fukunaga *et al.*, 1992). Interestingly, 8-epi-$PGF_{2\alpha}$ is considered to be an antagonist at the TXA_2 / PGH_2 receptor on both rat and human platelets (Morrow *et al.*, 1992a). The contrasting profile demonstrated by 8-epi-$PGF_{2\alpha}$ on these two preparations may be reconciled by the existence of TXA_2 / PGH_2 receptor subtypes, or the presence of a novel receptor on the rat vasculature at which 8-epi-$PGF_{2\alpha}$ is an agonist and, like the TXA_2 / PGH_2 receptor, SQ29548 is an antagonist.

It has been reported that these non-cyclooxygenase - derived PGs, in contrast to those formed as result of cyclooxygenase activity, are essentially stored, being formed *in situ* on phospholipids (Morrow *et al.*, 1992b). They are then released, possibly by the action of phospholipases. A new synthetic approach using carbohydrates has been developed for the chemical synthesis of these epi-PGs (Rokach *et al.*, 1992a,b). The finding that elevated plasma levels and an increased urinary excretion of these prostanoids occurs in hepatorenal syndrome (Moore *et al.*, 1992) suggests they may be involved in its pathogenesis.

1.2 Control of prostanoid biosynthesis and action

In 1971 it was discovered that aspirin and similar drugs inhibited the biosynthesis of PGs (Vane, 1971) and this was believed to be the mechanism of their therapeutic efficacy. There are two stages in the pathway of prostanoid synthesis which are susceptible to inhibition by anti-inflammatory agents. Most commonly used are the non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin (Kocsis *et al.*, 1973; Flower, 1974). Their mechanism of action is believed to be through the inhibition of cyclooxygenase, though they have been reported to show a differential profile of activity on each of the two forms of cyclooxygenase previously described (Meade *et al.*, 1992). NSAIDs thereby prevent the formation of PGG_2 from arachidonic acid, and consequently the PGs and TXs, though not the LTs (Vane & Botting, 1987; Robinson 1989). In fact by inhibiting cyclooxygenase activity, NSAIDs may actually increase LT synthesis through a build up of arachidonic acid being channelled into the lipoxygenase pathway.

Unwanted side-effects accompanying NSAID treatment may therefore arise as a result of the accompanying increased production of these LTs (Lee *et al.*, 1986; Robinson *et al.*, 1986). In addition, gastric ulceration is one of the major side-effects of NSAID therapy, occurring through inhibition of the formation of cytoprotective PGs (Reeves & Stables, 1985). It may therefore be more appropriate to inhibit the synthesis or action of particular PGs or TXs, to maintain synthesis of the beneficial prostanoids and prevent redirection of arachidonic acid to the potentially deleterious LTs.

Cyclooxygenase inhibitors such as aspirin have shown some anti-thrombotic activity in the clinic, however the simultaneous inhibition of endothelial cyclooxygenase presents a theoretical limitation to its usefulness. TXA_2 is one of the most potent inducers of platelet activation, and is involved in the development of thrombosis (Gresele *et al.*, 1991). Platelets, producing primarily TXA_2, are more susceptible to the inhibitory effects of NSAIDs as compared to the endothelium, which produces PGI_2. This is

thought to be due to the fact that 60 % of the aspirin is deacetylated to salicylate during first-pass metabolism, so that absorbed aspirin affects platelet cyclooxygenase whilst platelets are in the hepatic portal circulation, but the systemic plasma aspirin concentration is then too low to affect PGI_2 synthesis. In addition, platelets cannot regenerate cyclooxygenase since they have no nucleus (Steiner, 1970), formation of new platelets from bone marrow stem cells, which themselves are sensitive to aspirin, being required to restore cyclooxygenase activity. A single dose of aspirin may therefore have an effect on platelet function lasting well over a week.

Since PGI_2 is a potent inhibitor of platelet activation, it may be of benefit to preserve its production whilst inhibiting that of TXA_2 in conditions such as thrombosis. Given the differential sensitivity of their production to NSAIDs, it was hoped to develop a regimen which allowed the formation of PGI_2 in the absence of TXA_2. Since much of the PGH_2 for PGI_2 synthesis is formed in the platelet, this is an impossible task as any inhibition of platelet cyclooxygenase would effectively reduce endothelial cell PGI_2 production (Patrono, 1989). Efforts were then concentrated on developing a TX synthetase inhibitor to selectively prevent the conversion of PGH_2 to TXA_2. It was reasoned that the PGH_2 destined for TXA_2 formation would then be redirected and consequently not only would PGI_2 synthesis be spared, it would actually be enhanced. In addition it was expected that the platelet levels of antiaggregatory PGD_2 would also be raised. However, the PGH_2 accumulated may also interact with the platelet and vascular wall TXA_2 / PGH_2 receptor, thereby inducing vasoconstriction and platelet aggregation and opposing the inhibitory effect of additional PGI_2 synthesised (Gresele *et al.*, 1984,1987).

The alternative use of a TXA_2 / PGH_2 receptor antagonist alone has its limitations since it may be displaced from the receptors by high concentrations of TXA_2 generated at the localised sites of platelet activation. Also whilst PGI_2 production would be preserved, it would not be enhanced as in the presence of a TX synthetase blocker, nor would there be any effect on aggregation induced by

TXA_2-independent stimulators of platelet aggregation, such as high dose collagen or thrombin. Perhaps a combination of a TX synthetase inhibitor and a TP-receptor antagonist would overcome the problems arising due to administration of the TX synthetase inhibitor alone and improve on the effect of TXA_2 / PGH_2 receptor antagonists (Gresele *et al.*, 1987). The resulting increase in cyclic AMP as a result of such a combination may suppress platelet aggregation induced by other stimuli, thereby potentially reducing both TXA_2- dependent and independent platelet activation. Examples of such drugs include ridogrel (De Clerck *et al.*, 1989a,b; Hoet *et al.*, 1990), picotamide (Gresele *et al.*, 1989) and D1542 (Brewster *et al.*, 1992; Brownlie *et al.* 1992; Snow *et al.*, 1992).

All actions of the NSAIDs are however, not the result of inhibition of prostanoid synthesis. 7-Oxa-13-prostynoic acid, indomethacin and the mefenamates inhibited the effects of PGE_1 and PGE_2 in elevating cyclic AMP levels in human astrocytoma cells (Ortmann & Perkins, 1977). The fenamates have been reported to exert this additional anti-inflammatory effect at the level of the receptor (Rees *et al.*, 1988; Rees & Bernal, 1989; Bernal *et al.*, 1991). In particular, meclofenamic acid demonstrated weak but specific blocking activity at prostanoid EP_1- and TP- receptors, as demonstrated by the antagonism of PGE_2- and U 46619 - induced contraction of the guinea-pig fundus and rat aorta respectively (Head *et al.*, 1992). Meclofenamate was also shown to inhibit PGE binding and adenylyl cyclase activation in the human myometrium (Bernal *et al.*, 1991). In addition, at concentrations greater than those required for inhibition of prostanoid synthesis, NSAIDs such as aspirin, ibuprofen, indomethacin, piroxicam and sodium salicylate, have been demonstrated to exert an anti-inflammatory effect through inhibition of neutrophil activation (Rampart & Williams, 1986a; Weissman, 1987; Abramson & Weissmann, 1989).

Corticosteroids also inhibit production of the prostanoids though they act at an earlier step in the pathway than do NSAIDs, namely the release of arachidonic acid from phospholipid. The

mammalian group II PLA_2 is found in inflammatory exudates and is the form susceptible to the inhibitory effect of the corticosteroids. Several mechanisms have been reported to be involved. This inhibitory activity was initially demonstrated on perfused guinea-pig lungs, and since then the release of PGs from various tissues has been shown to be reduced, but not the conversion from exogenous arachidonic acid (Gryglewski *et al.*, 1975). In contrast to the NSAIDs, steroids also inhibit production of the LTs, since these too are derived from arachidonic acid, though by the action of lipoxygenase.

Recently it has been suggested that corticosteroids, besides their PLA_2-inhibitory activity, also inhibit PG synthesis at the level of cyclooxygenase (Goppelt-Struebe *et al.*, 1989), and that the lack of effect of the corticosteroids on conversion of arachidonic acid to PGs previously reported may be a result of inappropriate time periods. Dexamethasone was shown to significantly inhibit prostanoid synthesis from exogenous arachidonic acid in bone marrow - derived macrophages by an effect on cyclooxygenase and / or PGE synthetase. Whilst having an effect on endogenous PLA_2, the predominant effect of dexamethasone was on the conversion of arachidonic acid to its metabolites.

The effect of the steroids through inhibition of arachidonic acid release is possibly mediated through the formation of an inhibitory protein, which binds to PLA_2 thereby preventing its action. Several of these inhibitory proteins have been reported, such as macrocortin (Blackwell *et al.*, 1980), lipomodulin (Hirata *et al.*, 1980) and renocortin (Cloix *et al.*, 1983). The term lipocortin is now used collectively for such inhibitory proteins (Di Rosa, 1984; Flower, 1985,1988; Rothwell & Flower, 1992). Since corticosteroids predominantly alter gene expression, they may block PLA_2 - messenger RNA (mRNA) synthesis, and in addition have been reported to inhibit post-transcriptional expression of group II PLA_2 (Nakano *et al.*, 1990). A similar inhibition of translational and post-translational cyclooxygenase activity has also been suggested (Flower, 1992). Such an effect has been demonstrated, in the *in vivo* models of human and rat

arthritis (Sano *et al.*, 1992). In these inflammatory joint diseases, there was a corticosteroid-sensitive upregulation of cyclooxygenase expression. It has been reported that the mRNA and protein of the second cycloxygenase gene cloned is that form of the enzyme sensitive to this inhibition by corticosteroids (Herschman, 1992; O'Banion *et al.*, 1992; Smith & Marnett, 1992). The ability of dexamethasone to selectively down-regulate the expression of this gene is thought to occur by a non-transcriptional mechanism involving destabilisation of the corresponding mRNA (Simmons *et al.*, 1992).

Corticosteroids therefore appear to exert their anti-inflammatory effect both at the level of release of arachidonic acid and its conversion to the prostanoids. Possible mechanisms include inhibition of transcription, and post-transcriptional effects such as destabilisation of mRNA and synthesis of inhibitory proteins. Lipocortins have been cloned and sequenced (Wallner *et al.*, 1986) and found to be related to a family of calcium and phospholipid binding proteins, calpactins and calelectrins (Davidson *et al.*, 1987). The finding that inhibition by lipocortin is substrate concentration dependent raises the possibility that they may in fact act by binding to phospholipid and not PLA_2 as proposed (Aarsman *et al.*, 1987).

Regulation of nutritional lipids is an alternative to the control of eicosanoid production besides such inhibition of enzyme activity. The most abundant essential fatty acid in the Western diet is arachidonic acid, which may also be formed by desaturation and chain elongation of dietary linoleic acid. As discussed, this fatty acid gives rise to the 2-series prostanoids. Dietary γ-linolenic and oleic acid are metabolised to dihomo-γ-linolenic acid, forming monoenoic prostanoids, and eicosapentaenoic acid, forming trienoic prostanoids, respectively. There is competition between these three dietary fatty acids for desaturases responsible for the synthesis of tissue polyunsaturated fatty acids. Enrichment of the diet with the latter two, may therefore increase the formation of the 1- and 3- series prostanoids, at the expense of the 2-series. An inhibitory influence is also observed at the levels of

cyclooxygenase and lipoxygenase, where linolenic acid inhibits the oxygenation of arachidonic acid by cyclooxygenase (Belch *et al.*, 1988; Hwang, 1989) and dihomo-γ-linolenic acid forms a derivative which blocks the transformation of arachidonic acid into the leukotrienes.

Animal studies have shown diets rich in either primrose oil, increasing the levels of γ-linolenic acid, or eicosapentaenoic acid, both exert an anti-inflammatory effect (Tate *et al.*, 1988). In man, ingestion of evening primrose oil or eicosapentaenoic acid have been shown to be of benefit in conditions of eczema and rheumatoid arthritis. Indeed, incorporation into the diet of evening primrose oil alone or in combination with fish oil, which contains eicosapentaenoic acid, allowed some patients to reduce or stop their treatment with NSAIDs. This influence of dietary fatty acids in conditions of inflammation should prove to be useful where patients suffer peptic ulceration or renal impairment, thereby precluding the use of NSAIDs.

1.3 Prostanoid Receptors

Several properties of the prostanoids suggest the existence of prostanoid receptors, including :
- high potency (Oien *et al.*, 1975);
- small chemical modifications having profound effects on their potency and profile of biological activity (Pike *et al.*, 1967);
- different prostanoids having different effects on the same cell type (Moncada *et al.*, 1976).

Distinct receptors for each of the five different naturally occurring prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, TXA_2, were consequently proposed. These have been designated DP, EP, FP, IP and TP, where the letter preceding P, for prostanoid, refers to the natural prostanoid having the greatest potency at that receptor.

Direct evidence for prostanoid receptors initially resulted from work conducted by Pickles in 1967, studying the effects of a series of PGE and PGF analogues on the guinea-pig uterus, human

myometrium and rabbit jejunum. This was supported by a later study into the structure-activity relationships for analogues of PGA, PGE and PGF (Andersen & Ramwell, 1974). Indeed, a binding study suggested a PGE receptor to be present on rat adipocytes (Kuehl & Humes, 1972). The initial classification by Gardiner & Collier (1980) suggested three types of prostanoid receptor, ψ, λ and ω, based on the results of a range of natural and synthetic agonists in airways. Following a comparison of the potencies of PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and the stable TX-mimetic, U46619 (Coleman *et al.*, 1981), distinct receptors were proposed for each (Kennedy *et al.*, 1982, 1983; Coleman *et al.*, 1984; Coleman, 1988). This work has been supported both pharmacologically (Jones *et al.*, 1984; Tynan *et al.*, 1984; Whalley & White, 1980) and biochemically (Jones *et al.*, 1984; Robertson, 1986). It is this later classification upon which prostanoid receptor nomenclature is now based. PG receptors have been classified more recently based on their coupling to signal transduction systems (Muallem *et al.*, 1989). This group suggests that tissue response measurement may not be representative of the interaction of the agonist with its receptor, especially where there is more than one PG receptor mediating the same response, and specific antagonists for each of the naturally occurring PGs are not available. An analysis of the results from both methods on a common tissue would be of interest, to determine to what extent the profiles differ.

Prostanoid receptor classification does not stop here though, further studies having identified subtypes of the DP, EP and TP receptors. Since this thesis is concerned with the characterisation of EP-receptors, the evidence leading to its subdivision into the EP_1-, EP_2-, EP_3-, and EP_4- receptors (Coleman *et al.*, 1985a; Coleman 1987; Coleman *et al.*, 1987a,b,c; Rao, 1988) will be presented.

Bennet & Posner (1971) first suggested that two subtypes of PGE receptor may exist. This resulted from their finding that SC 19220 and polyphloretin phosphate blocked the contractile action of PGE_2 on the longitudinal muscle of the guinea-pig ileum,

whereas the relaxant action of PGE_2 on the circular muscle was unaffected. Since then, Coleman *et al.* (1980) found SC 19220 to be inhibitory to PGE_2 on only some of a range of EP-receptor-containing tissues, such as the guinea-pig ileum and fundus, but not the guinea-pig lung, dog saphenous vein, rabbit aorta, dog and cat iris or cat trachea. EP-receptors which were blocked by SC 19220 were designated EP_1-receptors. The effectiveness of this compound was limited by its low potency and solubility, subsequently being superseded by AH 6809 (Coleman *et al.*, 1985a) with a similar profile of action but having both a greater potency and solubility.

This was supported by another study conducted by Dong *et al.* (1986), this time involving a comparison of the effects of the EP-receptor agonists ICI 80205, 16,16-dimethyl PGE_2 and 11-deoxy PGE_1 with that of PGE_2. Whilst ICI 80205 and 16,16-dimethyl PGE_2 were more active than PGE_2 as contractile agents on bullock iris sphincter, rat stomach fundus and guinea-pig trachea, PGE_2 was the more potent relaxing agent on cat and guinea-pig trachea, and dog hind limb arterial vessels *in vivo*. 11-Deoxy PGE_1 however showed more relaxant than contractile potency, when compared to PGE_2.

This subclassification of the EP-receptor has been confirmed and extended by the identification of the selective agonists, sulprostone and AY 23626. These two compounds showed a different profile of activity on tissues not sensitive to the activity of SC 19220 or AH 6809, leading to the division of the non-EP_1 EP-receptors into two further subtypes, EP_2 and EP_3 (Coleman *et al.*, 1987a,b,c). Sulprostone has a high potency at EP_1- and EP_3- (chick ileum) receptors, and is inactive at EP_2-receptors (cat trachea), whereas AY 23626 is potent at EP_2- and EP_3- receptors, having weak or no activity at EP_1-receptors. Similarly, Coleman *et al.* (1987c) using the guinea-pig vas deferens and guinea-pig ileum circular muscle preparations, found an effect of PGE_2 which was not blocked on either by SC 19220 or AH 6809. Sulprostone on the other hand was shown to be highly active as an inhibitor of the twitch

response in field stimulated guinea-pig vas deferens, whereas it was inactive in relaxing the guinea-pig ileum circular muscle. The action of sulprostone in inhibiting the histamine-induced acid secretion of the rat isolated gastric mucosa showed a similar potency to that on the vas deferens described above, and its action was unaffected by SC 19220 (Reeves *et al.*, 1988).

The use of such analogues having differing selectivity for each of these three EP-receptor subtypes and the EP_1 antagonists, have allowed the identification of the EP-receptor subtype(s) in a given system to be established. Table 1.1 outlines the EP-receptor subtypes present in various tissues, which may then be used to determine the EP-receptor subtype activity of novel compounds.

EP_1- and EP_3- receptors in general mediate contraction of smooth muscle and EP_3-receptors also mediate inhibition of transmitter release. This may involve a rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$, (Creese & Denborough, 1981) or a fall in intracellular levels of cyclic AMP (Gutman *et al.*, 1979). Meanwhile, the relaxation of smooth muscle is generally mediated by EP_2-receptors. It is likely that this effect is the result of an increase in intracellular cyclic AMP levels, such an increase accompanying the PGE_2-induced relaxation of vascular and intestinal smooth muscle (Creese & Denborough, 1981).

More recently the possibility of a fourth EP-receptor subtype has been reported as indicated in the table referred to above. An investigation of the EP-receptor subtype mediating relaxation of the rabbit jugular vein (Lawrence & Jones, 1992) suggested it to be similar to the EP_2-receptor, but the putative EP_2-selective agonist butaprost showed a low potency on this preparation as compared to the cat trachea. The hamster uterus preparation (Yeardley *et al.*, 1992) enforced this requirement for a fourth subtype. Whilst PGE_2 inhibited uterine contractions and increased intracellular levels of cyclic AMP, AH 13205, EP_2-selective, and sulprostone, EP_1 and EP_3- selective, were inactive in these respects. Having found AH 13205 and sulprostone to be 6,500 times and 11,000 times respectively less active than PGE_2 in relaxing pre-contracted pig saphenous vein, Louttit *et al.* (1992a)

suggested the existence of an EP_4-receptor. In addition, the TP-receptor blocking drug AH 23848B, whilst demonstrating no antagonistic activity on guinea-pig fundus (EP_1-receptor preparation), rabbit ear artery (EP_2-receptor preparation) or guinea-pig vas deferens (EP_3-receptor preparation), was reported to have a weak antagonistic activity, $pA_2 = 5.36$, at this 'new' receptor subtype (Louttit *et al.*, 1992b).

Many PGE analogues have been synthesised, showing differing selectivity for the EP_1-, EP_2- and EP_3- receptor subtypes (see Figures 1.2 & 1.3; structures of additional compounds used are given in Figure 1.4). The activity of these analogues on the putative EP_4-receptor subtype is as yet not known, since the possibility of its existence is only now emerging. For evidence of the selectivity of the PGE analogues the reader is referred to the following references :

AH 13205	Nials *et al.*, 1991.
AH 23848B	Louttit *et al.*, 1992b.
AH 6809	Coleman *et al.*, 1985a.
butaprost	Gardiner, 1986.
11-deoxy PGE_1	Dong *et al.*, 1986.
11-deoxy PGE_2 1-alcohol	Lawrence *et al.*, 1992.
16,16-dimethyl PGE_2	Dong *et al.*, 1986 Coleman, 1987 Coleman *et al.*, 1988.
GR 63799X	Bunce *et al.*, 1990.
MB 28767	Banerjee *et al.*, 1985.
misoprostol	Bauer, 1985 Collins *et al.*, 1985 Coleman *et al.*, 1988 Eglen & Whiting, 1988 Tsai *et al.*, 1987, 1991.

17-phenyl-ω-trinor PGE_2	Johnson *et al.*, 1980 Dong *et al.*, 1986 Lawrence *et al.*, 1989,1992.
sulprostone	Schillinger *et al.*, 1979 Coleman *et al.*, 1987a,b, 1988 Lawrence *et al.*, 1992.

There are many prostanoid agonists which are analogues of PGE, but their biological actions are not necessarily mediated through EP-receptors. For instance, 16,16-dimethyl PGE_2, ICI 80205, MB 28767 and Wy 17186 all have TP agonist activity on vascular smooth muscle and blood platelets which may be blocked with GR 32191 (Lumley *et al.*, 1989), whilst PGE_1 has been proposed to act on IP-receptors (Miller & Gorman, 1979). Similarly, it is not essential that an agonist be a PGE analogue for it to behave as an EP agonist. Considering the stable prostacyclin analogue iloprost, besides showing potent IP agonism on both platelets and vascular smooth muscle, it is also a potent agonist at EP_1-receptors (Dong *et al.*, 1986; Sheldrick *et al.*, 1988).

To date, only EP-receptor antagonists specific for the EP_1-receptor subtype have been developed, and include the earlier mentioned SC 19220 and AH 6809 (Coleman *et al.*, 1985b). These two compounds each have their limitations. The poor aqueous solubility and low potency of SC 19220 limit its usefulness *in vivo*. Although AH 6809 has a higher solubility and potency compared to SC 19220, it also blocks DP- and TP- receptors (Keery & Lumley, 1988) at higher concentrations, and is highly protein bound (Coleman *et al.*, 1985c). Diphloretin phosphate (DPP) also possesses some EP_1-receptor blocking activity, though like AH 6809 is also believed to have antagonistic activity at both DP- and TP-receptors. In contrast to the above mentioned EP_1-receptor antagonists, 13,14-didehydro-20-methyl carbaprostacyclin is structurally related to the prostanoids, being a PGI_2 analogue (Fassina *et al.*, 1985). It has been shown to inhibit the effect of PGI_2 on guinea-pig tracheal smooth muscle with a pA_2 of 7.2. Since this preparation contains no IP-receptors, the

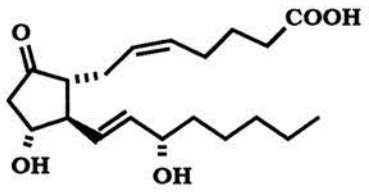
activity of PGI_2 is likely to be mediated by EP_1-receptors. In the absence of more selective antagonists then, we are currently reliant on the PGE analogues for characterisation of the EP-receptor subtype in a given system.

There has been some recent success in the cloning of prostanoid receptors. Having cloned cDNA for both the human and mouse TP-receptors (Hirata *et al.*, 1991), whose respective proteins demonstrated 78 % homology, cDNA for the mouse EP_3-receptor subtype was then isolated and expressed by the same group (Sugimoto *et al.*, 1992). The cloned PGE receptor showed 30 - 40 % homology to the TP-receptors, and has been shown to couple to G_i. Whilst such molecular cloning techniques are valuable in differentiating between receptors and their subtypes, they must be complemented with functional studies to demonstrate the biological relevance of molecular differences.

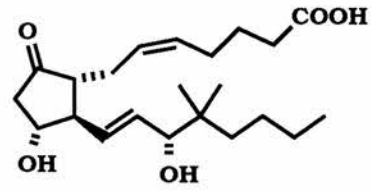
Prostanoids have been implicated in many pathological conditions including inflammation (Lewis, 1983), cancer (Goodwin *et al.*, 1980), pyrexia (Milton & Wendlandt, 1971), destruction of cartilage and bone (Lewis, 1983), thrombosis and occlusive vascular disease (Hirsch *et al.*, 1981), renal disease (Scharschmidt *et al.*, 1986), asthma (Horton, 1969) and migraine (Lance, 1973). The development of drugs which interfere with their synthesis or action is therefore of potentially great therapeutic value. Consequently classification of prostanoid receptors and their subtypes is important in order that drugs be developed which retain the beneficial effects to the exclusion of those that are adverse.

Figure 1.1 The biosynthesis of prostanoids

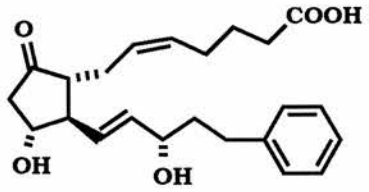
Figure 1.2 Specificity of PGE analogues



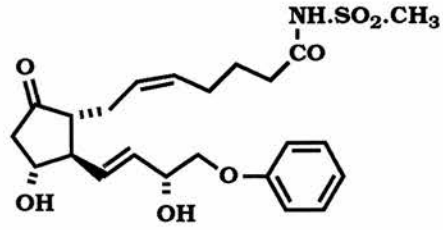
PGE_2



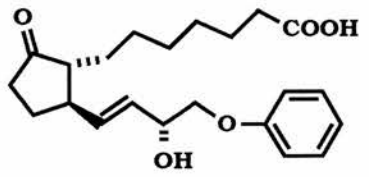
16,16-dimethyl PGE_2



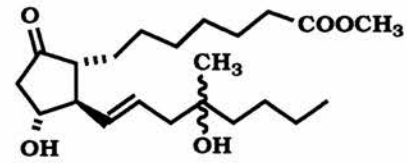
17-phenyl-ω-trinor PGE_2



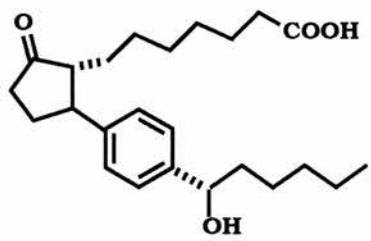
Sulprostone



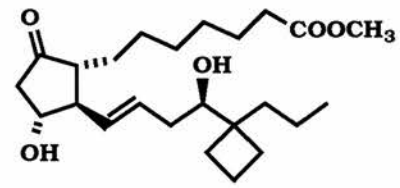
MB 28767



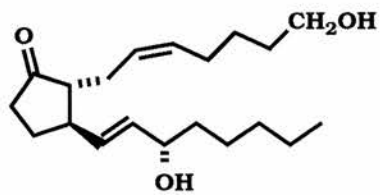
Misoprostol



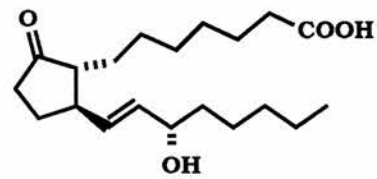
AH 13205



Butaprost

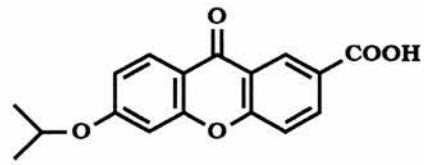


11-deoxy PGE_2-1-alcohol

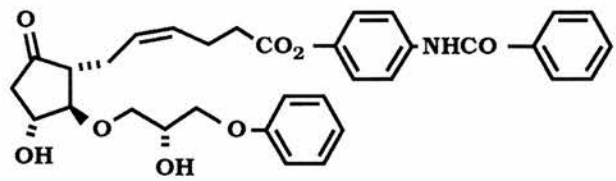


11-deoxy PGE_1

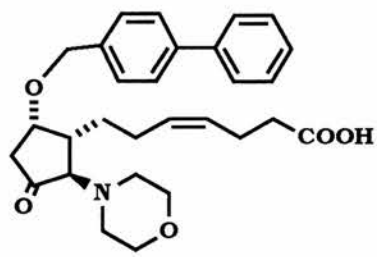
Figure 1.3 Structures of PGE analogues



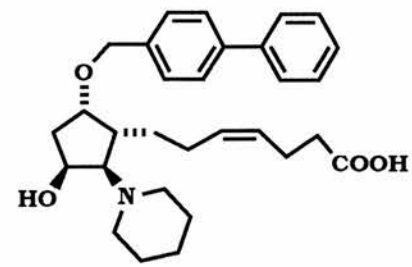
AH 6809 - EP_1 antagonist



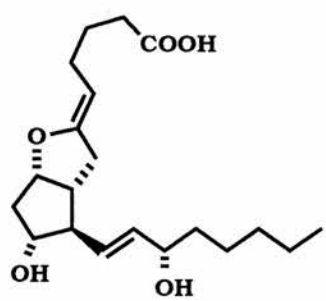
GR 63799X - EP_3 selective agonist



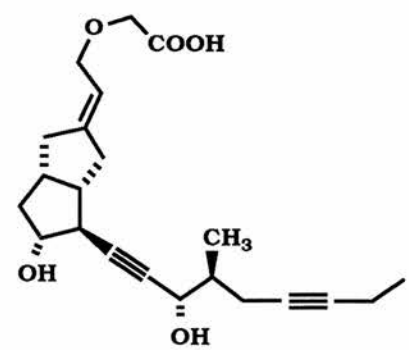
AH 23848 - EP_4 selective antagonist



GR 32191 - TP-receptor antagonist



PGI_2 - IP-receptor agonist



Cicaprost - stable IP-receptor agonist

Figure 1.4 Further structures

Table 1.1 EP-receptor subtypes and tissues expressing them.

Smooth muscle contraction is typical of EP_1-receptor activation :

guinea-pig ileum	(Coleman *et al.*, 1985a,b; Coleman, 1987)
guinea-pig fundus	"
dog and cat fundus	(Coleman, 1987)
rat and pig fundus	"
guinea-pig trachea	(Coleman & Kennedy, 1985)
human myometrium	(Senior *et al.*, 1991)

Smooth muscle relaxation is typical of EP_2-receptor activation :

guinea-pig trachea	(Coleman & Kennedy, 1985)
cat and human trachea	(Gardiner, 1986; Coleman *et al.*, 1987c)
guinea-pig ileum	(Coleman *et al.*, 1985a)
rabbit jugular vein	(Coleman, 1987)
dog saphenous vein	"
human myometrium	(Gardiner, 1986; Clayton *et al.*, 1986)
cat ciliary muscle	(Chen & Woodward, 1992)

The following are typical of EP_3-receptor activation :

inhibition of:

twitch of guinea-pig vas deferens (Hedqvist & Von Euler, 1972)

release from adrenergic nerve terminals (Mantelli *et al.*, 1991)

contraction of:

chick ileum	(Coleman *et al.*, 1987c)
rat and pig fundus	(Coleman, 1987)
rabbit renal artery	(Ahluwalia *et al.*, 1988)
guinea-pig uterine artery	(Baxter *et al.*, 1989)
human myometrium	(Senior *et al.*, 1991)

The following may involve putative EP_4-receptor activation :

relaxation of :

rabbit jugular vein	(Lawrence & Jones, 1992)
hamster uterus	(Yeardley *et al.*, 1992)
pig saphenous vein	(Louttit *et al.*, 1992a,b)

inhibition of :

macrophage interleukin 1 (IL-1) release (Chapter 4, this thesis)

Chapter 2

Characterisation of the PGE receptor mediating the pro-inflammatory effect of PGE_2 in a rabbit model of skin inflammation

2.1 INTRODUCTION

2.1.1 The inflammatory response

Inflammation is the body's reaction to injury, and is characterised by redness, heat, pain and swelling. These symptoms are caused by an increased blood supply to the infected area, an increased capillary permeability and migration of leucocytes out of the capillaries and into the surrounding tissue. Once in the tissue, these cells migrate to the site of infection in response to concentration gradients of inflammatory mediators, a process known as chemotaxis. The polymorphonuclear (PMN) leucocytes (neutrophils, eosinophils and basophils) are the first of the blood leucocytes to enter the area of the inflammatory reaction, followed several hours later by the mononuclear leucocytes (monocytes and lymphocytes). These leucocytes engulf tissue debris, dead cells and microorganisms and are important in the repair processes.

There are many mediators of inflammation including : amines (histamine and 5-hydroxytryptamine (5HT)); short peptides (bradykinin); long peptides (interleukin-1); lipids (prostaglandins and leukotrienes); enzymes released by migrating cells and complement. The increased capillary permeability caused by contraction of the endothelial cells, allows the larger of these molecules to cross the endothelium thereby gaining access to the site of infection from the blood. Mast cells containing histamine and 5HT are already present in the tissues.

The inflammatory reaction and concomitant immune response are a defensive mechanism against disease-causing organisms. The immune system confers specificity on the reaction, the inflammatory reaction representing an effector. Sometimes this defensive mechanism is raised to innocuous substances from outside the body, or against tissues of the body itself such as in asthma or rheumatoid arthritis. In these conditions anti-inflammatory or immunosuppressive drugs are of considerable value.

2.1.2 Prostaglandins in inflammatory exudation

PGE_2 was first identified in carrageenan-induced rat inflammatory exudate by Willis (1969a,b), and subsequently in human cutaneous inflammation, both as a result of exposure to ultraviolet light (Greaves & Søndergaard, 1970) and in the condition of allergic contact eczema (Greaves *et al.*, 1971). Since NSAIDs have been shown to inhibit prostaglandin synthesis in guinea-pig lung cell-free homogenates (Vane, 1971), isolated human platelets (Smith & Willis, 1971) and in perfused dog spleen (Ferreira *et al.*, 1971), with a potency comparable to that as inhibitors of carrageenan-induced oedema, PGs are thought to have an important role in inflammation. The significance of these findings has been enhanced by the demonstration that PGs can induce signs of inflammation both in rat and man (Crunkhorn & Willis, 1969,1971a), causing increased vascular permeability indirectly through degranulation of mast cells. The criteria for classifying a compound as a mediator of inflammation have therefore been met. These are: induction of the signs of inflammation, release of the candidate substance during an inflammatory reaction in a concentration capable of inducing inflammation, and reduction of release by known anti-inflammatory drugs.

Following i.v. administration of pontamine blue to the rat, the effect of intradermally injected PGs E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ was assessed both by measurement of the mean diameter of each blue reaction site, and by scoring their intensity on a five-point scale (Crunkhorn & Willis, 1971a). PGE_1 and PGE_2 showed a threshold effect on local vascular permeability at a dose of 1 ng, and at 100 ng produced a marked increase; their potency was comparable to other putative mediators of inflammation such as histamine, bradykinin and 5HT. $PGF_{1\alpha}$ and $PGF_{2\alpha}$ in microgram doses did not produce any significant effect themselves, though $PGF_{2\alpha}$ (500 ng) was shown to inhibit the increased permeability in response to 100 ng of either PGE_1 or PGE_2 (Crunkhorn & Willis, 1971b). This may reflect a local control of inflammation to minimise potential injury. Thomas & West (1973)

however, whilst suggesting that PGE_1 potentiates the response to bradykinin in the rat skin, found the response to bradykinin and PGE_2 to be only additive. Injecting the PGEs subcutaneously into the rat hindpaw gave a similar profile of action (Thomas & West, 1974).

Experiments carried out following i.v. administration of mepyramine (histamine H_1-receptor antagonist) or methysergide (5HT-antagonist), or following depletion of mast cell amines with compound 48/80, indicate PGE_2 exerts its potentiating effect in the rat through the release of mast cell amines, with the release of histamine being predominant (Crunkhorn & Willis, 1971a). Mepyramine and methysergide inhibited the cutaneous response to histamine and 5HT respectively, whilst compound 48/80 had no effect on either. None of the three treatments altered the response to bradykinin, whilst the PGE_2-induced increase in permeability was susceptible to all, mepyramine more so than methysergide. The effect of compound 48/80 itself in increasing local vascular permeability in rat skin was blocked by $PGF_{2\alpha}$ (500 ng). It was therefore suggested that $PGF_{2\alpha}$ may act by inhibiting the release of mast cell amines by both PGE_1, PGE_2 and compound 48/80. Although PGA_1 is of much lower potency than PGE_2, blueing occurring only at doses greater than 100 ng, it appears that it too exerts its effect through the release of histamine, since it could be blocked by pretreatment with mepyramine. Release of mast cell amines may therefore reflect a common mechanism for increasing local vascular permeability in the rat.

This cutaneous reaction to i.d. PGE_1 and PGE_2 has also been demonstrated in the rat hindpaw model of inflammation where the effect of PGs on other inflammatory mediators has also been studied (Di Rosa *et al.*, 1971). To assess the effect of PGs administered i.d., rats were treated with indomethacin to suppress endogenous prostaglandin formation, such pretreatment reducing the paw swelling induced solely by carrageenan, a phlogogen extracted from seaweed. 100 ng PGE_1 and PGE_2 were both shown to potentiate the rat paw swelling induced by carrageenan, with

PGE_1 being approximately 5 times more potent than PGE_2. The potentiation by PGE appeared to be fairly specific since $PGF_{2\alpha}$ (500 ng) gave no potentiation; histamine was only a weak potentiator with 20 μg being as effective as 0.1 μg of PGE_1 and bradykinin at 1 μg was ineffective. In another study (Lewis *et al.*, 1975) the volume of both hindpaws of each rat was measured by mercury displacement plethysmometry. PGE_1 and PGE_2 produced a mild paw swelling at 100 ng, and potentiated the response to 1 mg carrageenan, whilst $PGF_{2\alpha}$ (250 ng) proved to be ineffective both itself and in the presence of carrageenan.

This study was extended beyond the PGs themselves to their precursor, arachidonic acid, and linolenic acid, which is not a substrate for PG synthetase. Neither of these fatty acids produced any oedema themselves, and it was only arachidonic acid which potentiated the response to carrageenan. The effect of arachidonic acid was reduced by the PG synthetase inhibitors, indomethacin, naproxen and phenylbutazone, and may therefore be the result of its conversion to PGs, in particular PGE_2.

PGI_2 has also been shown to potentiate the carrageenan-induced rat paw swelling, though it was 10-fold less potent than PGE_1 (Komoriya *et al.*, 1978). This potentiation was not inhibited by the presence of the NSAIDs, dexamethasone or indomethacin, but was abolished by diphenhydramine (histamine-antagonist). Histamine is therefore thought to be involved in the PGI_2-induced potentiation of carrageenan. In indomethacin-treated rats, the smooth muscle relaxants PGE_1 and adenosine both potentiated the paw oedema response to carrageenan, whilst papaverine was ineffective in this respect (Thomas, 1980). PGE_1 and adenosine, unlike papaverine, were ineffective in inhibiting the response to noradrenaline in the perfused rat mesenteric circulation, therefore it was proposed that the potentiation of oedema was unrelated to vasodilatation. ^{133}Xe clearance studies carried out in rat skin may prove to be more relevant in assessing the involvement of vasodilatation in the action of these agents.

In the absence of pontamine blue dye, the results in man were not

as easily assessed (Crunkhorn & Willis, 1971a). PGE_1 and PGE_2 were however found to induce a cutaneous reaction at 25 - 100 ng, consisting of an initial wheal followed by a diffuse redness. $PGF_{1\alpha}$ or $PGF_{2\alpha}$ at 500 ng or 5 μg produced a more localised but more prolonged reaction, with no apparent potentiation of the response when used in combination with PGE. A similar sensitivity to PGE was therefore observed in rat and man, though no appreciable response to PGF was evident in the rat. Perhaps release of mast cell amines is the mechanism of action of PGE in man, as already reported for the rat. Another qualitative study demonstrated i.d. injection or infusion of PGE_2 into man produced an immediate weal and flare response (Ferreira, 1972). More recently a study of a more quantitative nature investigated the effect of i.d. injection of PAF and PGE_2 into the forearm of eight healthy male volunteers (Sciberras *et al.*, 1987). The area of the injection site and skin-fold thickness were used to determine flare and wheal responses. Both PAF and PGE_2 induced a dose-related effect.

Such a response to the PGs is however species-dependent, as in the guinea-pig (Williams & Morley, 1973) and rabbit (Ikeda *et al.*, 1975) PGs on their own cause virtually no increase in vascular permeability. They do however potentiate the response to mediators such as histamine or bradykinin, which act directly on the post-capillary venules to induce some oedema, and to FMLP, C5a and LTB_4, which themselves do not cause significant oedema but in the presence of a vasodilator induce oedema dependent on circulating leucocytes. Guinea pigs were administered i.v. ^{125}I-guinea-pig serum albumin, followed by i.d. injection of the inflammatory agent into the dorso-lateral skin. PGE_1 at 1 μg, which itself caused virtually no effect above saline, shifted the dose-response to bradykinin to the left, and increased the slope. PGE_1 at ng doses was also able to potentiate the response to 0.5 μg histamine, reflecting the physiological significance of this activity. The potentiation of bradykinin and histamine observed by the PGs has the following potency ranking: $PGE_1 > PGE_2 > PGA_2 > PGF_{2\alpha}$. In this study PGE_1, at ng doses, was also shown to potentiate endogenous inflammatory reactions such as Type I

(immediate or anaphylactic hypersensitivity, eg. hayfever, asthma, urticaria), Type III (immune complex - mediated hypersensitivity, eg. Arthus' reaction which occurs if a foreign protein is injected subcutaneously into a rabbit or guinea pig with a high concentration of circulating antibody against that protein), and Type IV (cell-mediated hypersensitivity, eg. mumps, measles) reactions.

Potentiation of bradykinin not only by PGE_2 but also by arachidonic acid has been demonstrated in rabbit skin (Ikeda *et al.*, 1975). Pontamine blue was administered i.v., and the diameter of the blue skin areas measured. This protocol suggested both PGE_1 and PGE_2 alone to be equipotent to bradykinin. However, if the dye was extracted, the PGE_1 and PGE_2 activities were shown to be 20 times less active at inducing plasma exudation when compared to bradykinin, and $PGF_{1\alpha}$ and $PGF_{2\alpha}$ 20 times less active again than the PGE response. Diffusion of the dye in the areas of skin receiving PG injections was responsible for the misleading results obtained initially by comparing diameters of the injection sites.

Despite the slight response to PGE_2 alone, it potentiated the response to bradykinin (100 ng), 100 ng PGE_2 increasing the response 10-fold, and 1 μg increasing it 100-fold. Arachidonic acid (1 μg) was also able to potentiate the response to bradykinin, an effect which was inhibited by indomethacin. Indomethacin had no inhibitory effect on the potentiation of the bradykinin response to PGE_2 nor on bradykinin or histamine alone. Arachidonic acid itself produced an increase in vascular permeability, which was less than the response to PGE_2 alone, and may be a result of its conversion to PGE_2 since the effect was inhibited by indomethacin. This was supported by the activity of the antagonist to PG action diphloretin phosphate, in significantly inhibiting the higher doses of arachidonic acid in increasing vascular permeability.

The arachidonic acid effect was markedly inhibited by previous administration of pyrilamine (mepyramine in Britain). Whilst histamine may be partly responsible for the effect of arachidonic acid alone, it is unlikely to be involved in the potentiating effect on bradykinin, since histamine and bradykinin in combination were

shown to be only additive.

2.1.3 Does vasodilatation account for the action of the prostaglandins ?

Dilatation, through the sequence of increased flow, increased supply to the venules and increased hydrostatic pressure, may increase the leakage of plasma proteins. In order to assess the importance of vasodilatation in the potentiation of oedema responses, a method for simultaneous measurement of plasma exudation and blood flow was developed in the rabbit (Williams, 1976a) using ^{131}I-albumin accumulation and ^{133}Xe washout respectively. The ^{131}I-albumin is administered i.v. through an ear vein, the accumulated counts being representative of plasma exudation, whereas ^{133}Xe is injected i.d. at each site and ln (natural logarithm) residual radioactivity is inversely proportional to local blood flow.

The rabbit skin inflammation model was used to study the relationship between vasodilatation and potentiation of plasma exudation by a series of prostaglandins and their analogues (Williams, 1976b). The results obtained supported a link between the two. Considering the PGs E, A, D, F and the analogues 16,16-dimethyl PGE_2 and 15(S)-15-methyl PGE_2, the rank order of exudation potentiating activity of the prostaglandins correlated with their vasodilating potency. The PGEs gave prolonged dilatation in the skin, so that even when histamine was administered 30 min after PGE_2, the potentiation was still in parallel with the observed increase in blood flow.

Also, other vasodilators such as ADP, isoprenaline (Kenawy *et al.*, 1978) and adenosine caused significant potentiation of the histamine responses. VIP, possibly released endogenously from mast cells, PMN leucocytes or cutaneous nerves, was able to increase local blood flow, and whilst causing little plasma exudation itself, potentiated the response to both bradykinin and C5a (Williams, 1982). Indomethacin had no effect on this action of VIP, which was more potent than that to PGE_2. Even more potent

again is the activity of pituitary adenylate cyclase activating polypeptide (PCAP) (Warren *et al.*, 1992). As with the other modulators of plasma exudation described, whilst alone having no significant effect on microvascular leakage, PCAP was shown to be a potent dilator and potentiator of bradykinin-induced oedema, being 100 times more potent than VIP.

It was also observed that addition of vasoconstrictors such as angiotensin II or noradrenaline (Kenawy *et al.*, 1978) to histamine-PG mixtures before i.d. injection, reduced blood flow and exudation in parallel. Williams (1979) expanded on the study by Ikeda *et al.* (1975), by measuring not only the increases in plasma exudation induced by the PGs and arachidonate, but also the effects of these agents on local blood flow. PGE_2, PGI_2, PGD_2 and $PGF_{2\alpha}$ alone produced little plasma exudation in the rabbit dorsal skin. However, PGE_2 and PGI_2 were potent at increasing blood flow and potentiated plasma exudation with similar relative potencies, whilst PGD_2 and $PGF_{2\alpha}$ increased local blood flow and potentiated the response to bradykinin only at non-physiological doses. Arachidonate alone, like the PGs themselves, gave little plasma exudation but increased blood flow and potentiated the response to bradykinin. A similar effect of PGE_2 in the rabbit model of acute pulmonary inflammation underlines the importance of vasodilatation in its pro-inflammatory effect (Downey *et al.*, 1988).

2.1.4 The two mediator hypothesis

The vasodilatation and plasma exudation components of inflammation may be considered as separate entities, as demonstrated in rabbit skin (Williams, 1977a). Bradykinin was shown to increase vascular permeability though it was much less potent at increasing blood flow, the reverse being reported for PGE_2 (Williams & Peck, 1977). In combination, a greater plasma exudation than that in the presence of bradykinin alone was induced. The potentiation of the bradykinin response to arachidonic acid was suppressed by locally injected indomethacin, that to PGE_2 remaining unaltered. This is consistent with the

proposition that indomethacin suppresses inflammatory dilatation and as a consequence inflammatory oedema, by preventing the production of a vasodilator substance via the cyclooxygenase pathway. Whilst indomethacin blocked the increase in blood flow induced by arachidonic acid, it had very little effect on basal blood flow, and so the production of vasodilator substances from endogenous arachidonic acid is considered to be low under normal conditions (Williams, 1979).

Further studies involving i.d. injection of *B. pertussis* vaccine, which itself induces plasma exudation and an increase in blood flow, support the two mediator hypothesis (Williams & Peck, 1977). The administration of indomethacin suppresses both features of the *B. pertussis* response. The presence of the endogenous vasodilator mediator is indicated by injecting bradykinin at the time of the peak response. The exudation response to bradykinin was potentiated as a result, this potentiation being sensitive to inhibition by indomethacin. By a similar method the presence of the endogenous permeability-increasing mediator was demonstrated. PGE_1 was injected again at the peak response to *B. pertussis*, and a potentiated exudation occurred which was unaffected by the presence of indomethacin.

In a similar manner the time-course of plasma exudation induced by zymosan (100 μg, i.d.), a yeast cell wall preparation, has been followed both in the presence and in the absence of PGI_2 (100 ng) (Williams, 1979). Since the level of PGI_2 is much greater than any endogenous vasodilator, the time-course of exudation is taken to reflect the changes in the levels of the permeability increasing mediator. Whilst exudation to zymosan alone was insignificant in the first 30 min, marked potentiation was observed in the presence of PGI_2, indicating the production of a permeability-increasing mediator, in the absence of endogenous vasodilator. The permeability-increasing mediator reached its peak at 30 - 60 min but exudation to zymosan did not reach its maximum until after this time period, when presumably the vasodilator component peaked.

These studies are the basis for proposing that inflammation may involve the separate production of permeability-increasing mediators and vasodilator mediators, production of the latter being susceptible to inhibition by indomethacin. Having established a mechanism by which PGs could potentiate other inflammatory mediators, an endogenous source of the natural PGs remained to be identified.

2.1.5 PMNs provide the PGs

In vitro phagocytosing rabbit peritoneal PMN leucocytes can produce PGs at 10 ng / 10^6 cells (56 % PGE_2; 28 % $PGF_{2\alpha}$; 16 % unknown) in response to killed bacteria, compared to < 0.3 ng / 10^6 cells in their absence (Higgs & Youlten, 1972). The PMNs can also convert exogenous arachidonic acid to PGs *in vitro* (McCall & Youlten, 1973). Since there is an influx of these leucocytes into inflammatory sites, an attempt was made to correlate this influx with the finding in rabbits that exogenous prostaglandins can potentiate inflammatory exudation *in vivo* (Williams, 1976b). The experiment involved the i.d. injection of PMN leucocytes into rabbit skin followed by bradykinin, blood flow and plasma exudation being measured by ^{133}Xe clearance and ^{131}I-albumin accumulation respectively (Firrell *et al.*, 1976). It was found that the PMN leucocytes (5 x 10^6 / site) potentiated the bradykinin-induced plasma exudation, and that this potentiation was inhibited when indomethacin (1 μg / site) was included along with the PMN leucocytes. The blood flow changes were consistent with the results of experiments using exogenous PGs (Williams, 1976b). The response of exogenous bradykinin is therefore thought to be potentiated by endogenous PG(s), the source of which is the PMN leucocytes.

Further evidence supporting PMN leucocytes to be the source of PG comes from studies using PMN leucocyte-depleted rabbits. Pretreatment with nitrogen mustard depletes circulating PMN leucocytes by preventing replication of stem cells in the bone marrow (Williams, 1977b). In these studies there was a marked

reduction in *B. pertussis* potentiation of plasma exudation induced by bradykinin, with no significant reduction in PGE_2 potentiation. Isolation of PMN leucocytes, and i.a. or i.v. injection into the previously PMN leucocyte - depleted rabbit, did not however restore responsiveness to *B. pertussis*. Injection of whole blood containing PMN leucocytes proved to be more successful, suggesting isolation of the cells has a harmful effect on their physiological function. Potentiation was also suppressed by mixing *B. pertussis* with indomethacin or dexamethasone before injection. These results suggest the infiltrated PMN leucocytes are the source of vasodilator, possibly a PG, which potentiates the exudation produced by other inflammatory mediators.

Studies have shown however, that even when there is little or no migration of PMN there is already a release of PGs by local cells (Glatt *et al.*, 1974), and that this release potentiates the effects of other mediators (Moncada *et al.*, 1973). This source of PGs may be the platelets (De Gaetano *et al.*, 1989), mast cells and mononuclear cells. Subsequent phagocytosis by PMN would then release additional PGs, contributing further to the potentiation. Indeed these may be the major source of the PGs, since NSAIDs were shown to be most effective in carrageenan-induced oedema of the rat hindpaw in the later stages of the development of the oedema, coinciding with the influx of leucocytes (Di Rosa *et al.*, 1971). Assay of the mediators in inflammatory exudate produced by carrageenan in the rat showed the release of histamine and bradykinin in the early phase, with PGE_2 being released at 2-3 hr (Willis, 1969b). The levels of histamine and PGE_2 then rose steadily and paralleled the time-course of migration of the PMN leucocytes into the site of injury (Di Rosa *et al.*, 1971). The inhibitory effect of indomethacin on carrageenan-induced oedema, demonstrated by Moncada *et al.* (1973), was apparent from as early as 1 hr when there was little or no influx of PMN leucocytes, suggesting a release of PGs by local cells potentiating the effect of other mediators.

2.1.6 Directly and indirectly acting inflammatory mediators

The permeability-increasing mediator in response to zymosan, is thought to be derived from blood plasma and has been termed a plasma activation permeability agent (PAPA) (Williams, 1978). Zymosan (1 mg/ml) was added to fresh heparinised rabbit plasma at 37 °C, and removed by centrifugation after 30 min. The ability of the resulting activated plasma (P*) to cause plasma exudation following i.d. injection, was then tested by measuring the accumulation of i.v. administered ^{131}I-albumin. PGE_1 and P* alone each produced very little plasma exudation, and whilst PGE_1 with plasma produced only a small increase, PGE_1 in combination with P* increased the volume of exudate to more than 10 times that of either alone. This latter response was comparable to that obtained in the presence of histamine and PGE_1 but was not susceptible to inhibition by the histamine antagonist, mepyramine. Zymosan therefore appears to induce a histamine-independent PAPA. The activity in the plasma has since been purified and identified as the cleavage product of the fifth component of complement, C5a (Williams & Jose, 1981).

Whilst histamine and bradykinin act directly on the venular endothelial cells to cause plasma exudation (Gabbiani *et al.*, 1970), C5a and FMLP have a different mechanism of action. The increase in vascular permeability in response to these latter agents cannot be induced in rabbits depleted of circulating neutrophils, whilst the responses to histamine and bradykinin are not affected (Wedmore & Williams, 1981). The LTs have also been shown to exert an effect on plasma exudation, which varies depending on the LT and the species under investigation (Williams, 1983). Dual inhibitors of both PG and LT production may therefore have benefits over existing drugs in controlling inflammatory conditions. LTC_4 and LTD_4 increase the vascular permeability in guinea-pig skin, as measured by Evans blue dye extravasation, but their constrictor component restricts oedema. LTB_4 on the other hand has very little activity in the guinea-pig skin, but in the presence of PGE_2 it increases oedema formation in rabbit skin, where LTC_4 and LTD_4 are inactive. LTB_4, like C5a and FMLP, is highly

chemotactic (Salmon & Higgs, 1987) and in neutrophil-depleted rabbits it has no effect.

PMN cells have a phagocytic role at the site of inflammation, where activation by these agents results in activation of the arachidonic acid cascade, generation of superoxide anions and release of lysosomal enzymes. They are also involved in the control of fluid efflux through the blood vessel wall leading to tissue oedema. LTB_4, C5a and FMLP have been shown to significantly enhance the binding of human PMN cells to selected populations of human dermal microvascular endothelial cells *in vitro* (Reusch *et al.*, 1988). These neutrophil-dependent mediators may open the junctions between the endothelial cells by inducing swelling and aggregation of the neutrophils, as has been demonstrated *in vitro* for both rabbit peritoneal and human blood neutrophils (O'Flaherty *et al.*, 1978).

Bradykinin reproduces several aspects of the inflammatory processes, including increased capillary permeability, stimulation of eicosanoid synthesis and induction of connective tissue proliferation. Unlike many of the other biologically active peptides, it is synthesised at the site of its action by plasma or tissue kallikrein acting on several circulating kininogen substrates, and is also rapidly degraded at its site of action by kininases. The involvement of both histamine and the cyclooxygenase products in the activity of bradykinin is controversial. Bradykinin given i.v. increased the plasma levels of both 6-oxo-$PGF_{1\alpha}$ and 13,14-dihydro-15-oxo-$PGF_{2\alpha}$, metabolites of PGI_2 and PGE_2 respectively, in conscious rabbits, and was inhibited in doing so by aspirin (Warren *et al.*,1987). Bradykinin is believed to stimulate the release of PGE_2 and PGI_2 from non-endothelial structures, probably smooth muscle cells, as removal of the endothelium did not significantly decrease their formation (Førstermann *et al.*, 1986). The PGs so formed relaxed isolated rabbit blood vessels, and both the formation and relaxation were sensitive to inhibition by indomethacin. Terfenadine, a

histamine-antagonist, had no effect on the wheal and flare response to bradykinin, and the response was only slightly inhibited by aspirin, suggesting neither histamine nor cyclo-oxygenase products are responsible for these cutaneous reactions (Crossman & Fuller, 1988). This is in accordance with the observation that co-injected indomethacin did not alter blood flow or plasma exudation in response to bradykinin in the rabbit.

A previous report did however show significant inhibition of the increased vascular permeability response to bradykinin in rabbit skin by the histamine-1 antagonist, mepyramine, though no modification of the response by indomethacin was observed (Marceau *et al.*, 1981). In view of the fact that bradykinin can stimulate the production of PGs, and these are known to potentiate the response to bradykinin in the rabbit as already reported, the lack of effect of indomethacin in the response to bradykinin alone is surprising. Murine macrophages and lymphocytes may also be stimulated by bradykinin to produce inflammatory cytokines, which cause proliferation of connective tissue and stimulation of both eicosanoid and proteolytic enzyme release (Tiffany & Burch, 1989).

2.1.7 The PG-inflammatory model is not a simple one

It has been proposed that PGE and PGF do not always mimic the inflammatory reactions, and the involvement of the PG endoperoxide PGG_2 should be considered (Kuehl *et al.*, 1977). MK-447 (2-aminomethyl-4-t-butyl-6-iodophenol) proved to be a good anti-inflammatory agent in rat hindpaw oedema, being 1.6 times more potent than phenylbutazone, though 6 times less potent than indomethacin. The differing slope of its dose-response curve compared to phenylbutazone and indomethacin, suggested it had a different mechanism of action. It was indeed found to stimulate PGE and PGF synthesis, in contrast to the PG synthetase inhibiting effect of the other two agents. Since both inhibitors and stimulators of PGE and PGF synthesis

were found to be anti-inflammatory, an intermediate in their formation was thought to be important. PGG_2 may serve this purpose, its action being blocked by the PG synthetase inhibitors, and also by the MK-447 - facilitated conversion to PGE and PGF. Williams & Peck (1977) have suggested that the capacity of arachidonic acid - derived products to provoke modest oedema, and to potentiate oedema induced by bradykinin and histamine reflects an enhanced vasodilatation. Using isolated vascular strips a two-phase vascular response to PGG_2 was observed, with an initial constriction followed by a strong dilatation (Bunting *et al.*, 1976). A similar effect was obtained on microvessels *in vivo*, using the hamster cheek pouch preparation (Lewis *et al.*, 1977). In agreement, ^{133}Xe studies in rabbit dorsal skin demonstrated an increase and then decrease in local blood flow, with vasodilatation predominating (Lewis *et al.*, 1977). It is suggested the potentiation of the bradykinin response may be by PGG_2 itself, or indirectly through conversion to its products.

The complex nature of the inflammatory process is demonstrated by the finding that systemic administration of the PGEs leads to anti-inflammatory responses. This has been shown in the rat (Fantone *et al.*, 1980), where PGE_1 or its stable analogue 15(S)-15-methyl-prostaglandin E_1 given i.v., both markedly reduce the increase in vasopermeability induced by i.d. injection of histamine, 5HT, bradykinin, C3a or compound 48/80. This was not thought to be the result of hypotension or acute stress, and electron microscopy showed the inhibition to be associated with preservation of the tight junctions between the endothelial cells.

Systemic administration of 15(S)-15-methyl-prostaglandin E_1 has in fact been demonstrated to inhibit the chemotactic factor FMLP in activating rat PMN, an effect associated with the decreased binding affinity of [^{3}H]-FMLP to a specific receptor on the neutrophil plasma membrane (Fantone *et al.*, 1983). This inhibition of the activation of PMN may explain, at least in part, the anti-inflammatory effects already described.
15(S)-15-Methyl-PGE_1 administered orally was also shown to suppress chronic adjuvant arthritis and acute

immune complex - induced vasculitis in rats in a dose-dependent manner (Kunkel *et al.*, 1981).

PGI_2 has been shown to stimulate or inhibit PMN-dependent plasma leakage depending on whether it is administered i.d. or i.v. (Rampart & Williams, 1986b). Locally administered PGI_2 markedly potentiated the effect on local oedema induced by C5a des Arg, LTB_4, bradykinin and histamine. This enhancing effect of PGI_2 on the PMN-dependent oedema induced by C5a des Arg and LTB_4 was reversed following i.v. administered PGI_2. There was no effect on the oedema induced by histamine or bradykinin. Similarly, the oedema induced by FMLP was suppressed, but not that induced by PAF. Initially, 15-methyl PGE_1 given systemically selectively suppressed PMN-dependent oedema, but a threshold was reached above which the inhibition of oedema was no longer selective and was associated with a fall in systemic arterial blood pressure. The site of PG generation and action is therefore important in determining the influence on oedema formation.

Another anti-inflammatory activity of the PGs has been demonstrated in a model of mast-cell dependent inflammation of the hamster cheek pouch, evoked by antigen challenge (Raud *et al.*, 1988). Indomethacin inhibited the antigen-induced vasodilatation and potentiated the plasma extravasation, leucocyte accumulation and histamine release. This effect was due to inhibition of cyclooxygenase activity, since indomethacin inhibited the arachidonic acid induced vasodilatation without influencing the action of histamine or acetylcholine. Topical application of PGE_2 reversed the action of indomethacin.

Contrary to its inhibition of the antigen-induced reactions, PGE_2 potentiated the response to histamine and LTC_4. Since the antigen response was mast-cell dependent, PGE_2 may reduce the response through inhibition of mediator-release from the mast-cell. This study therefore demonstrates a dual action of locally administered PGE_2 in the hamster cheek pouch, by inhibition of mediator release and enhancement of mediator action. Following the mast-cell dependent inflammation as a result of antigen challenge, the effect of PGE_2 on mediator release

predominates, in an anti-inflammatory fashion. A greater emphasis on one or other of these mechanisms of action of PGE_2 in different models of inflammation, may in part be responsible for conflicting effects reported for NSAIDs and the PGs.

This study has more recently been extended to compare the influence of PGI_2 with PGE_2 on inflammation, induced by both antigen-challenge and histamine, and to assess the involvement of increased cyclic AMP, through forskolin-activated adenylate cyclase, or increased cyclic GMP, through nitroprusside-activated guanylate cyclase, in the activity of these PGs (Raud, 1990). All four agents increased local blood flow, though forskolin and nitroprusside were not as active as PGE_2 or PGI_2. Topical administration of PGE_2 or PGI_2 in vasodilator concentrations suppressed the plasma leakage induced by antigen-challenge, whilst potentiating that to histamine. Whilst nitroprusside potentiated the responses to both antigen and histamine, suggesting vasodilatation in itself was not responsible for the anti-inflammatory properties of the PGs, forskolin produced a similar profile of activity to PGE_2 and PGI_2. The anti-inflammatory effect of these latter two agents may therefore be the result of an elevation in cyclic AMP levels. This study highlights the dual activity of at least some PGs in inhibiting mediator release yet enhancing mediator action, the former resulting from a mechanism other than vasodilatation.

The PGE_2-induced potentiation of the bradykinin response in rabbit skin is complicated by the finding that *in vitro* PGE_2 is reported to act on at least four different subtypes, designated EP_1, EP_2, EP_3 and EP_4. In order to better understand the mechanism of this potentiation, PGE analogues of differing selectivity for each of the four receptor subtypes have been tested in this model, using both accumulation of ^{125}I-albumin as a measure of plasma exudation, and ^{133}Xe clearance for measurement of changes in local blood flow. Since vasodilatation is reported to be an important component of this potentiation, the EP_2-receptor subtype was considered to be the most likely candidate. This receptor is responsible for the vasodilator effects of PGE_2 in

several tissues, such as relaxation of cat and guinea-pig trachea and rabbit jugular vein *in vitro*, and dog hind limb vessels *in vivo*. A stable PGI_2 analogue, cicaprost, was also tested in order to assess the contribution of vasodilatation to the potentiation of the bradykinin response. The results suggested that vasodilatation may be too simple an explanation for the action of PGE_2 in this model of rabbit skin inflammation.

2.2 MATERIALS and METHODS

SOLUTIONS	Source
Sagatal	
Pentobarbitone sodium B.P. (Vet) 60 mg / ml diluted 1:2 in sterile saline	RMB Animal health Ltd. (PL 12/4040)
Heparinised-saline	
0.1 ml heparin sodium (5000U / ml) in 50 ml sterile saline	(BN 9004980)
Evans blue	
2.5 % in sterile saline	B.D.H. Chemicals
^{125}I-human serum albumin	
100 μCi ^{125}I-HSA	Amersham (IM 17P)
^{133}Xenon	
^{133}Xenon multidose 10 mCi / 10 ml	Amersham (XAS 110P)

CHEMICALS	Source
AH 6809	Dr. R.A. Coleman, Glaxo, U.K.
bradykinin	Cambridge Biochemicals (PP 05 2086B)
butaprost	Dr. P. Gardiner, Bayer, U.K.
cicaprost	Prof. Vorbruggen, Schering AG, Berlin
sulprostone	"
FMLP	Sigma (F 3506)
11-deoxy PGE_2-1-alcohol	Edinburgh (starting material *nat* PGA_2)
PGE_2	Cayman Chemicals, U.S.A.
17-phenyl-ω-trinor PGE_2	"
indomethacin	Sigma (I 7378)
misoprostol	Dr. P. Collins, G.D. Searle, U.S.A.
U46619	Upjohn Diagnostics U.S.A.

PROCEDURE

2.2.1 Plasma exudation studies

This protocol is based on a method by Williams & Morley (1973).

1. Male New Zealand white rabbits (1.7 - 4.7 kg; 2.9 ± 0.1 kg, n=44) were anaesthetised with 30 mg / kg sagatal i.v. through an ear vein, followed by an equal volume of heparinised saline to keep the line clear.

2. The whole of the back section was shaved and demarcated usually into 6 subsections, each containing 12 injection sites. In those cases where the injection number was increased to 15 in order to incorporate all necessary controls, the number of subsections was reduced to 4.

3. 5 μCi ^{125}I-HSA in 2 ml of 2.5 % Evans blue was administered through the line into the ear vein, again followed by heparinised saline.

4. After a period of 5 min, drug additions were administered rapidly by 100 μl i.d. injection into each site. Each addition was made to 4 or 6 injection sites according to the number of subsections, each of fixed site pattern. These replicate injections to each rabbit were representative of one experiment (n=1), and avoided bias from site and animal variation.

5. The rabbit was left for 30 min, before being killed by overdose of anaesthetic.

6. 10 ml of blood was taken by cardiac puncture and centrifuged at 740 g (2000 rpm) for 30 min.

7. The back skin was removed from the rabbit and blood pushed out of the vessels with a cold damp towel, to prevent inappropriate assessment of plasma exudation due to the presence of ^{125}I-albumin in the blood.

8. Discs of skin (16 mm) containing the injection sites were removed with a steel punch.

9. 3 x 1 ml aliquots of plasma were removed from the supernatant of the intracardiac blood sample.

10. Skin and plasma samples were then counted on automatic γ-counter.

Calculation

The DPM obtained for each injection site was related to μl plasma exudate, using the DPM per ml of plasma sample as a reference.

By simple proportion :

$$DPM_{\text{injection site}} = \frac{DPM_{\text{injection site}}}{DPM_{\text{plasma sample}}} \times 10^3 \ \mu l \text{ plasma exudate}$$

2.2.2 Blood flow studies

This protocol is based on a method by Williams (1976a).

^{133}Xe is useful for the determination of blood flow as it is negligibly bound to plasma protein and is itself inert. Due to its volatile nature however, certain precautions are required :
- syringes for drug additions must be primed with saline and kept on ice;
- injection sites must be punched out as soon as the skin is removed and paraffin oil added to each.

In these blood flow studies there was a limitation of 4 subsections each of 12 injection sites, and following addition of the drugs the rabbit was only left for 20 min. These restrictions were necessary in order to reduce variability of results due to the ability of ^{133}Xe to diffuse into surrounding tissue.

1. Male New Zealand white rabbits (2.1 - 4.0 kg; 3.0 ± 0.2 kg, n=11) were anaesthetised with 30 mg / kg sagatal i.v. through an ear vein, followed by an equal volume of heparinised saline to keep the line clear.

2. The whole of the back section was shaved and demarcated into 4 subsections, each containing 12 injection sites.

3. ^{133}Xe was added into each drug syringe behind perspex in a fume cupboard, and administered rapidly by 100 µl i.d. injection containing 5 - 10 µCi / site. Injections were made in quadruplicate in each rabbit, one per subsection, and were representative of one experiment (n=1), these multiple additions avoiding bias from site and animal variation.

4. 100 µl samples of the injection fluid were stored under paraffin oil in sealed tubes, to provide initial ^{133}Xe activity for clearance calculations.

5. 20 min after the injections the rabbit was killed by overdose of anaesthetic and the back skin removed.

6. Discs of skin (16 mm) containing the injection sites were then immediately removed with a steel punch, immersed in paraffin oil in polycarbonate tubes and capped.

7. Skin and injection fluid samples were then counted on an automatic γ-counter.

Calculation

Clearance of ^{133}Xe from the tissue is dependent on and a measure of the local tissue circulation.

% change in blood flow :

$$\frac{\ln {}^{133}Xe\ \text{count}_{\text{saline-injected skin}} - \ln {}^{133}Xe\ \text{count}_{\text{agent-injected skin}}}{\ln {}^{133}Xe\ \text{count}_{\text{0.1 ml injection fluid}} - \ln {}^{133}Xe\ \text{count}_{\text{saline-injected skin}}}$$

where ln = natural logarithm.

2.3 RESULTS

The vertical bars on the figures represent the s.e.mean. Differences in µl plasma exudation and % change in blood flow were assessed according to the Student's t-test, significance being accepted with values of probability less than 0.05.

PGE_2, a range of PG analogues and a TX analogue were tested in the *in vivo* model of rabbit skin inflammation over the dose range 1 ng to 5 µg. None of the drugs alone, at the highest dose tested in combination with bradykinin or FMLP, was able to induce plasma exudation significantly different from that to saline (Table 2.1). All drugs were given i.d.

Table 2.1 Effects of a series of agents on plasma exudation in rabbit skin.

Drug and dose	Volume of exudate (µl)	
	Drug treatment (n=3-4)	Saline control (n=3-4)
Butaprost 1 µg	17 ± 4	17 ± 4
Cicaprost 1 µg	12 ± 3	13 ± 2
11-Deoxy PGE_2 1-alcohol 5 µg	13 ± 2	16 ± 3
Misoprostol 1 µg	23 ± 6	16 ± 3
PGE_2 1 µg	18 ± 2	17 ± 2
17-Phenyl-ω-trinor PGE_2 1 µg	16 ± 2	19 ± 4
U 46619 1 µg	15 ± 4	17 ± 4

Bradykinin in 8 out of 10 experiments produced a response significantly different from that to saline (Table 2.2). In those two cases where significance was not achieved, there was one extreme value.

Table 2.2 Skin plasma exudate produced in response to i.d. injection of bradykinin as compared to saline.

Volume of exudate (µl)	
Bradykinin (0.5 µg) (n=3-12)	Saline (n=3-12)
28 ± 6 (p=0.084)	16 ± 3
47 ± 5	17 ± 2
49 ± 8	15 ± 5
52 ± 12	17 ± 4
55 ± 9	16 ± 3
57 ± 10	15 ± 2
57 ± 11	17 ± 4
60 ± 4	19 ± 3
68 ± 14	19 ± 4
85 ± 20 (p=0.061)	13 ± 1

As a control, in experiments which were conducted in the absence of a dose-response curve to PGE_2 in combination with bradykinin, a site was injected with 100 ng PGE_2 + 0.5 µg bradykinin. The results from such controls (133 ± 12 µl plasma exudate, representing an increase of 129 ± 11 % over bradykinin alone; n=19) were not significantly different from the values obtained in the dose-response studies (116 ± 13 µl plasma exudate, representing an increase of 163 ± 20 %; n=12).

2.3.1 *Time-course studies*

The time-periods used for measurement of plasma exudation and % change in blood flow were 30 min and 20 min respectively. Considering plasma exudation in the case of saline, bradykinin (0.5 μg) and PGE_2 or cicaprost (100 ng each) in combination with bradykinin, the responses to each were 93 %, 66 %, 65 % and 76 % of their maxima, as represented by 15 ± 5 μl, 49 ± 8 μl, 122 ± 17 μl and 147 ± 23 μl of plasma exudate respectively (Figure 2.1). The response to bradykinin in the absence or presence of PGE_2 or cicaprost was significantly different from saline, and the potentiation of the bradykinin response by PGE_2 or cicaprost significantly different from that to bradykinin alone.

The greatest % change in blood flow in response to either 0.1 ng (+ 30 ± 15 %) or 1 μg (+ 105 ± 22 %) PGE_2 was observed at 20 min after injection (Figure 2.2), that at 1 μg being a significant increase. Determination of the % change in blood flow is complicated by the presence of permeability-increasing mediators, therefore studies are conducted in the absence of bradykinin, when assessing vasodilator potencies.

2.3.2 *Potentiation and blood flow studies*

PGE_2 and cicaprost have both been shown to dose-dependently potentiate the response to 0.5 μg bradykinin, when administered in combination (Figure 2.3). A significant increase over the bradykinin alone response was observed from 0.1 ng PGE_2 and 0.3 ng cicaprost to the maximum dose of each tested of 1 μg. The results correlate well with those obtained in the previous time-course studies (Figure 2.1).

The exudation response to bradykinin was 47 ± 5 μl, PGE_2 alone (1 μg) 18 ± 2 μl, and in combination 109 ± 19 μl; this indicates that a more than additive response was obtained. The maximum % increase over the bradykinin response was 167 ± 29 % (113 ± 19 μl) at 100 ng PGE_2, and 231 ± 69 % (166 ± 31 μl) at 1 μg cicaprost, but it was only at the 1 ng dose of each that there was a significant difference between the responses to PGE_2 and cicaprost in combination with bradykinin.

Considering the % change in blood flow induced by PGE_2 and cicaprost alone (Figure 2.4), significance is achieved at 1 ng and maintained through to 1 μg. At 100 ng and 1 μg, the increase in blood flow induced by PGE_2 (107± 22 % and 110 ± 22 % respectively) is significantly greater than that resulting from cicaprost at the same doses (86 ± 21 % and 93 ± 23 % respectively). The results represented here for 0.1 ng (24 ± 16 %) and 1 μg (110 ± 22 %) PGE_2 in increasing % blood flow, correlate well with those obtained during the time-course studies (30 ± 15 %, 105 ± 22 % respectively) (Figure 2.2).

Another inflammatory mediator, FMLP, alone (5×10^{-11} moles / site; 22 ng) induced much lower plasma exudation, 18 ± 3 μl, (Figure 2.5) compared to that for bradykinin, 47 ± 5 μl (Figure 2.3), and the response was not significantly different from that to saline, 13 ± 2 μl. Whilst not significantly inducing plasma exudation themselves, PGE_2 and cicaprost, as in the presence of bradykinin, did however enhance the response to FMLP, being most significant at the lower doses with only PGE_2 maintaining significance at 1 μg (27 ± 5 μl = +108 ± 36 %). The responses to PGE_2 and cicaprost in the presence of FMLP reached levels not much more than additive, and were not significantly different from each other at any of the doses tested.

Butaprost (Figure 2.6) at 1 ng and 10 ng doses did not increase the response to bradykinin, and at 100 ng the increase was slight. Significance was achieved only at the highest dose tested of 1 μg, represented by 96 ± 17 μl, an increase of 94 ± 21 % over the bradykinin control.

The response to 11-deoxy PGE_2 1-alcohol is also represented in Figure 2.6. At 5 μg, 11-deoxy PGE_2 1-alcohol increased the bradykinin-induced plasma exudation from 28 ± 6 μl to 38 ± 6 μl, an effect which is at most additive. A significant difference from bradykinin alone was only achieved in the presence of 500 ng 11-deoxy PGE_2 1-alcohol, with 33 ± 7 μl representing an increase of 22 ± 4 %.

11-deoxy PGE_2 1-alcohol did not significantly alter local blood flow (Figure 2.7) up to the maximum dose tested of 5 μg. These studies were carried out in rabbits in which significant increases in blood flow were observed by PGE_2 and cicaprost at doses as low as 1 ng (Figure 2.4).

The effect of pretreatment with 11-deoxy PGE_2 1-alcohol (5 μg, 5 min) is demonstrated in Figure 2.8. The significant increase in plasma exudation over bradykinin achieved by 1 ng PGE_2, is absent following this pretreatment. Whilst the absolute values for PGE_2 (in the absence of 11-deoxy PGE_2 1-alcohol pretreatment) in combination with bradykinin were greater than those achieved previously (Figure 2.3), this may be accounted for by an increase in the bradykinin control response, 60 ± 4 μl here compared with 47 ± 5 μl in Figure 2.3. This is reflected by the similar % increases over bradykinin alone when comparing both sets of experiments.

11-deoxy PGE_2 1-alcohol pretreatment increased both saline and bradykinin controls, the latter achieving significance. In order to test for significant differences, the results following pretreatment were assessed relative to the 11-deoxy PGE_2 1-alcohol - bradykinin control. When expressed as % increase over the response to bradykinin alone, pretreatment with 11-deoxy PGE_2 1-alcohol doubled the response to 1 ng PGE_2 in combination with bradykinin from 33 ± 10 % to 76 ± 11 %, and increased the response slightly at 10 ng PGE_2 from 91 ± 10 % in the absence of 11-deoxy PGE_2 1-alcohol to 115 ± 17 % in its presence. The % increases at 100 ng + bradykinin and 1 μg PGE_2 + bradykinin were similar in the presence or absence of 11-deoxy PGE_2 1-alcohol pretreatment.

However, if expressed relative to the 11-deoxy PGE_2 1-alcohol - bradykinin control, which represents an increase of 66 ± 11 % over the response to bradykinin alone, the % increases are considerably less following pretreatment. Since the response to 11-deoxy PGE_2 1-alcohol pretreatment may have been additive, 10 μg PGE_2 + bradykinin was included in the experiment to check if the maximum response had been reached.

This combination gave 148 ± 49 µl plasma exudated, representing an increase of 144 ± 84 % over the response to bradykinin alone.

Preliminary studies carried out with 11-deoxy PGE_1 (n=2) showed a potentiation of the response to bradykinin. The response to 11-deoxy PGE_1 alone at 2.5 µg was 19 µl as compared to saline, 18 µl, whilst bradykinin alone resulted in 71 µl of plasma exudate. In combination with 2.5 µg 11-deoxy PGE_1, the response to bradykinin was potentiated to 145 µl, representing an increase of 104 % over the bradykinin control alone.

Misoprostol (Figure 2.9) potentiated the response to bradykinin, increasing the response significantly over that to bradykinin alone at doses of 100 ng and 1 µg. The maximum plasma exudation to misoprostol and bradykinin in combination was 156 ± 17 µl at 1 µg, which is an increase of 194 ± 30 %. At 1 ng and 10 ng doses, the responses to misoprostol + bradykinin were only marginally greater than that to bradykinin alone.

The influence of sulprostone and 17-phenyl-ω-trinor PGE_2 on the response to bradykinin, were tested in the presence and absence of AH 6809 (10 µM, 5 min pretreatment) (Figure 2.10). The response to bradykinin was not significantly altered by either sulprostone or 17-phenyl-ω-trinor PGE_2, nor the AH 6809 pretreatment. AH 6809 did however increase both the saline and bradykinin responses, that to saline being significantly increased in both the sulprostone and 17-phenyl-ω-trinor PGE_2 studies, whilst that to bradykinin was only significantly increased in the 17-phenyl-ω-trinor PGE_2 study, $p=0.070$ in the sulprostone study.

Preliminary studies into the effect of 17-phenyl-ω-trinor PGE_2 on a potentiated system (n=2), have also been carried out. PGE_2 at 100 ng increased the response of bradykinin to 148 µl plasma exudate, from 71 µl in its absence, an increase of 113 %. 1 µg 17-Phenyl-ω-trinor PGE_2 reduced this potentiation slightly to 138 µl (+ 93 %). Following pretreatment with AH 6809 (10 µM, 5 min), this slight fall in response was not apparent, though more experiments are required to assign any significance to this 17-phenyl-ω-trinor PGE_2 effect.

Studies with U 46619 were conducted to determine the influence of this TP-receptor agonist on a PGE_2-potentiated bradykinin response (Figure 2.11). In the absence of U46619, the response to 1 ng PGE_2 in combination with bradykinin (58 ± 13 µl) was small but significantly different from that to bradykinin alone (52 ± 12 µl). However, if the dose of PGE_2 was raised 100-fold, an increase of 161 ± 31 % over bradykinin alone was observed, represented by 124 ± 17 µl skin plasma volume. U46619 did significantly reduce the response of 100 ng PGE_2 + bradykinin to 90 ± 17 µl, 81 ± 23 % over bradykinin alone, a reduction of 28 ± 10 % of the control 100 ng PGE_2 + bradykinin response.

PGE_2 was shown to significantly increase the response to both 0.5 µg and 1 µg bradykinin (Figure 2.12). The bradykinin responses alone were not significantly different from each other, at 47 ± 5 µl and 55 ± 9 µl respectively. The PGE_2 responses in combination with bradykinin were only significantly different from each other at the 1 ng dose. The correlation between potentiation of the bradykinin response by 1 ng PGE_2 and the bradykinin response alone was tested and not found to be significant (Figure 2.13).

Preliminary studies with cicaprost in the presence of 1 µg bradykinin (n=2), demonstrated potentiation of the response (86 µl) at the lowest dose of cicaprost tested, 1 ng (144 µl, + 97 %). A dose-dependent increase in µl plasma exudated was obtained up to the maximum dose of cicaprost tested, 1 µg (197 µl, + 206 %). Whilst absolute values vary, the % increases over bradykinin alone are similar to those obtained with PGE_2 in place of cicaprost (Figure 2.12), and cicaprost with 0.5 µg bradykinin (Figure 2.3). More experiments would be required in order to statistically compare these results.

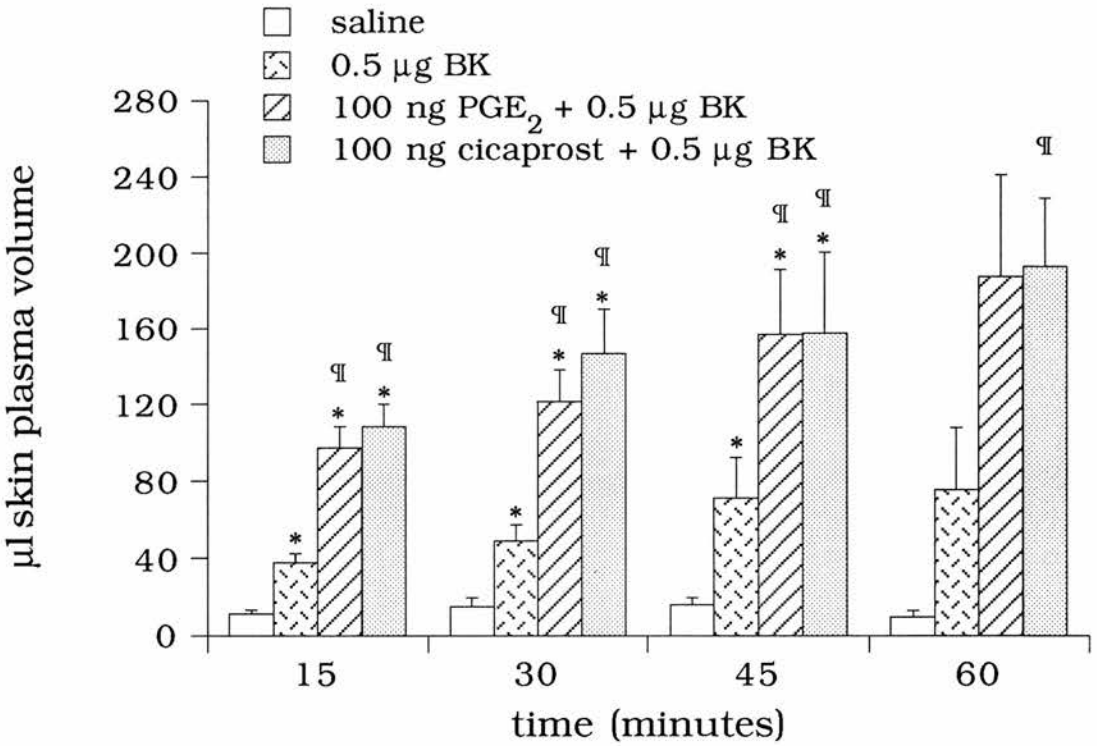


Figure 2.1 Time-course of µl plasma exudated in response to saline, bradykinin and PGE_2 or cicaprost in combination with bradykinin (n=4).

* denotes significant difference from saline response within the same time period ($p<0.05$).
¶ denotes significant difference from bradykinin response alone within the same time period ($p<0.05$).

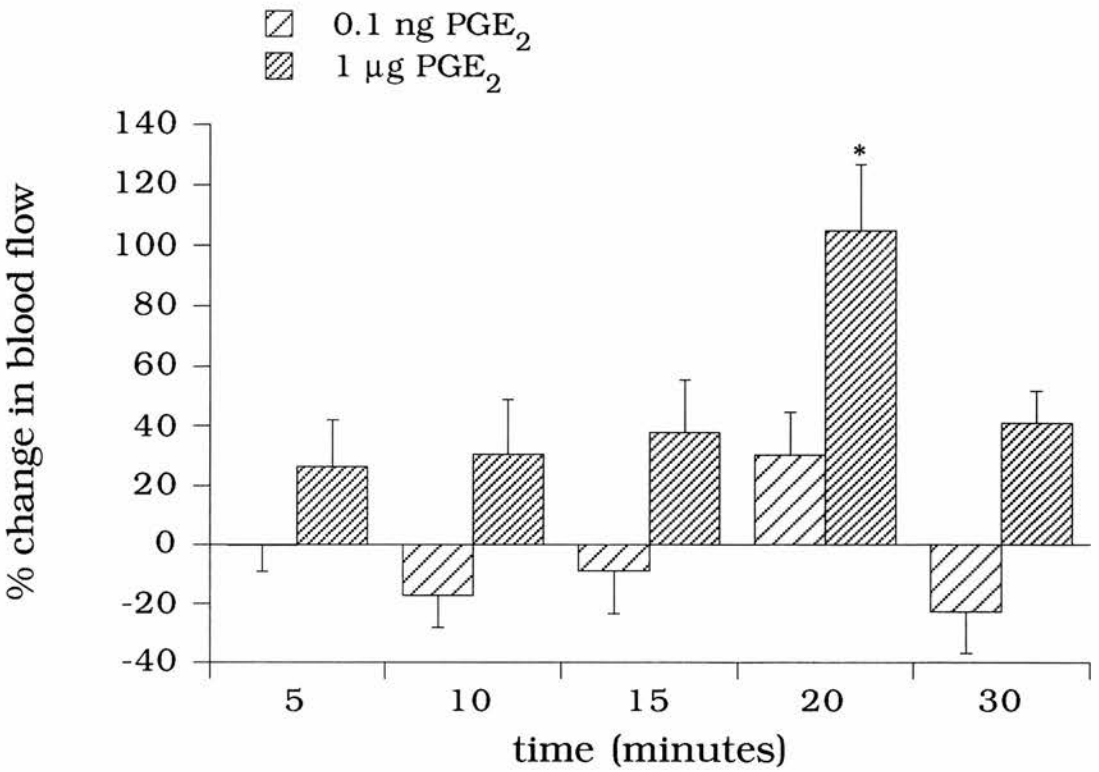


Figure 2.2 Time-course of % change in blood flow induced by low dose (0.1 ng) and high dose (1 µg) PGE_2 (n=4).

* denotes significant increase in blood flow ($p<0.05$).

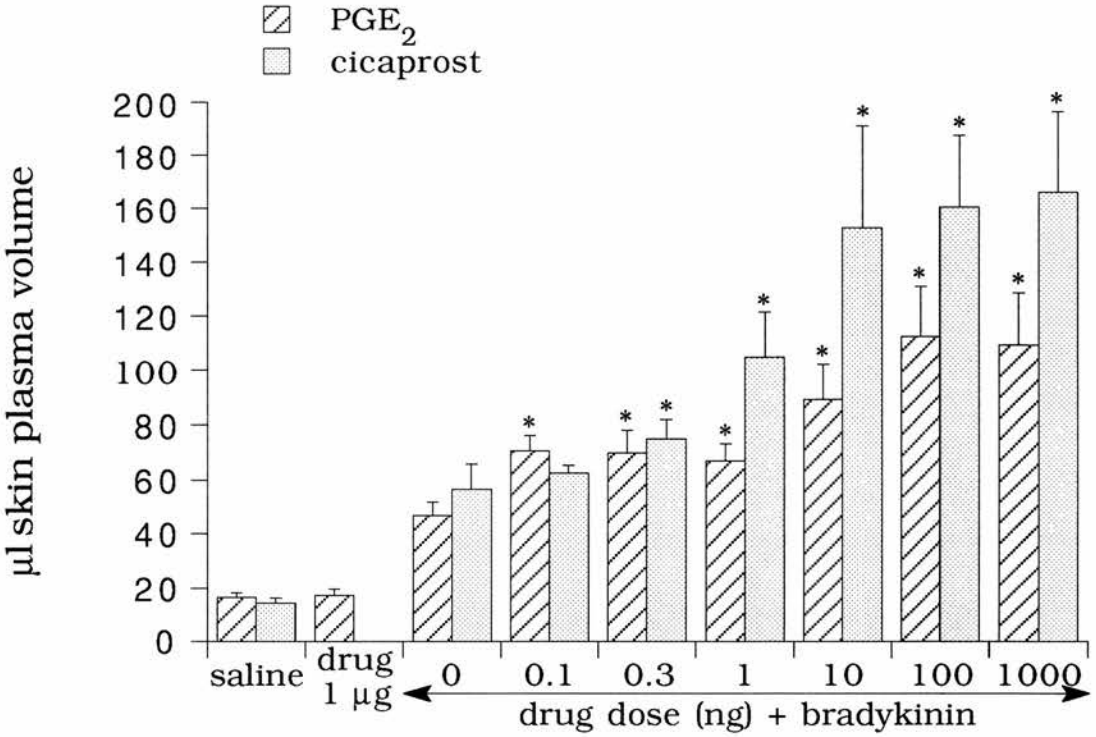


Figure 2.3 Dose-response to PGE_2 and cicaprost (n=4-12) in potentiating plasma exudation induced by 0.5 µg bradykinin.

* denotes significant difference from bradykinin response alone ($p<0.05$)

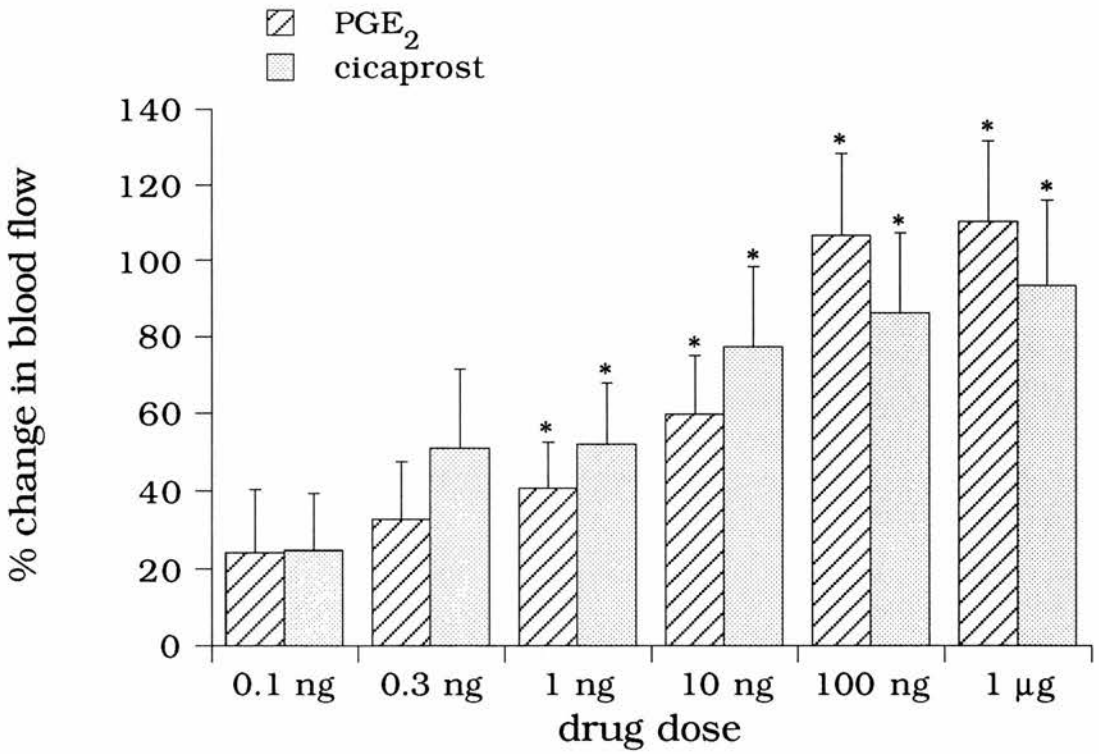


Figure 2.4 Dose-response comparing % change in blood flow induced by PGE_2 and cicaprost (n=4-10).

* denotes significant increase in blood flow ($p<0.05$)

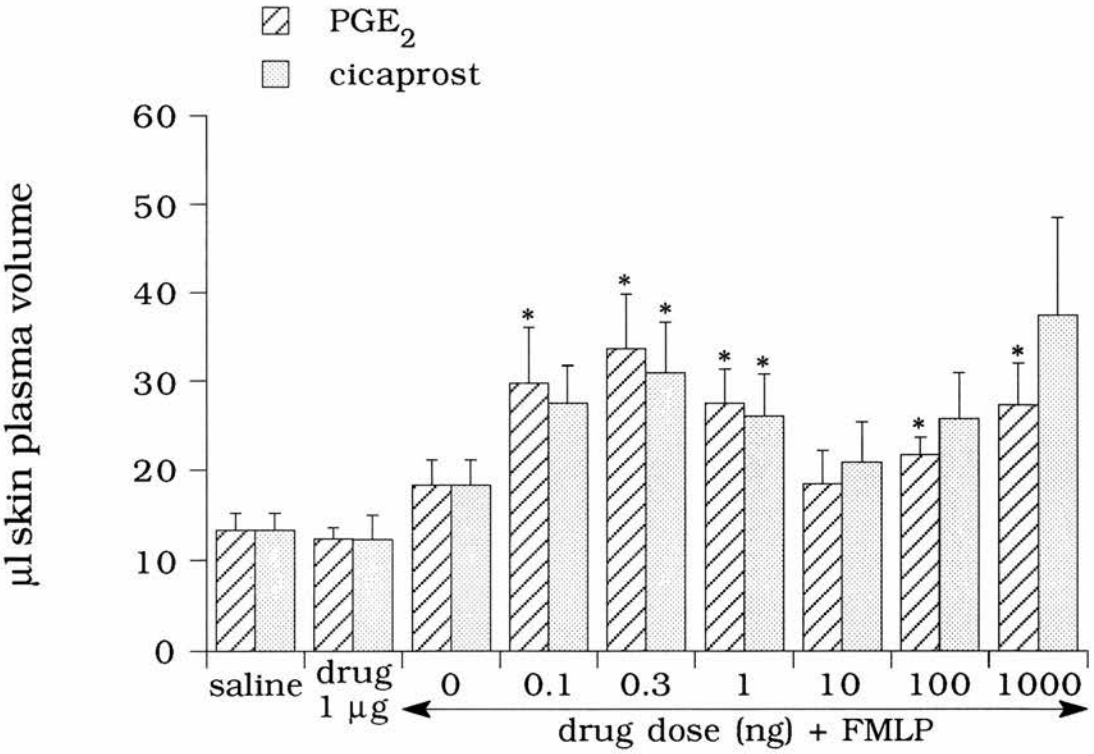


Figure 2.5 Dose-response (n=4-8) to PGE_2 and cicaprost in potentiating plasma exudation induced by 5×10^{-11} moles/site FMLP.

* denotes significant difference from FMLP response alone ($p<0.05$).

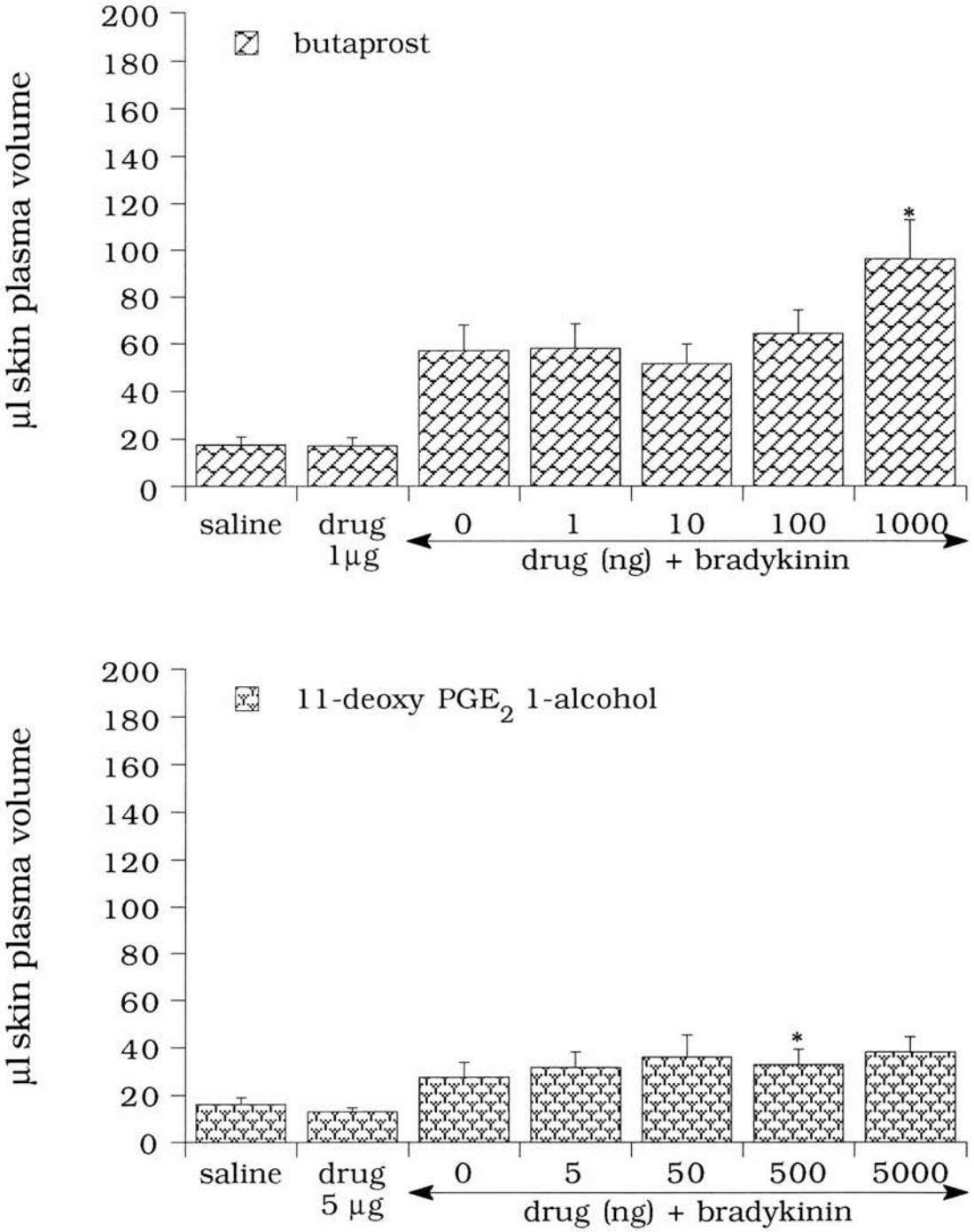


Figure 2.6 Potentiation of 0.5 µg bradykinin-induced plasma exudation by butaprost (n=3-4) and 11-deoxy PGE_2 1-alcohol (n=4).

* denotes significant difference from bradykinin response alone ($p<0.05$).

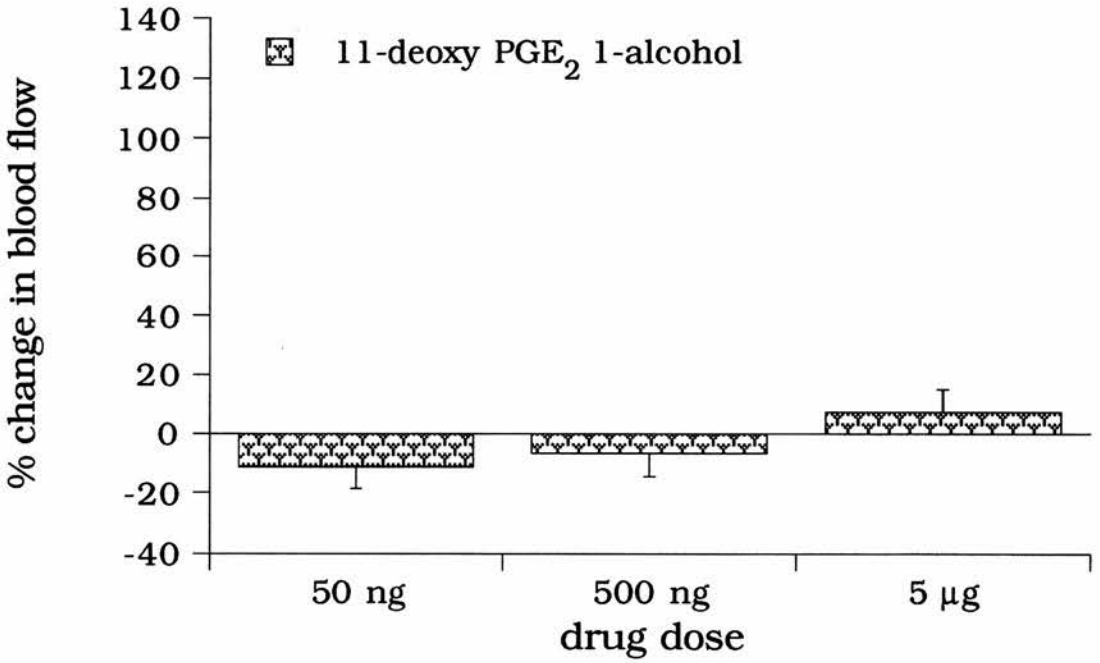


Figure 2.7 % change in blood flow induced by 11-deoxy PGE_2 1-alcohol (n=4).

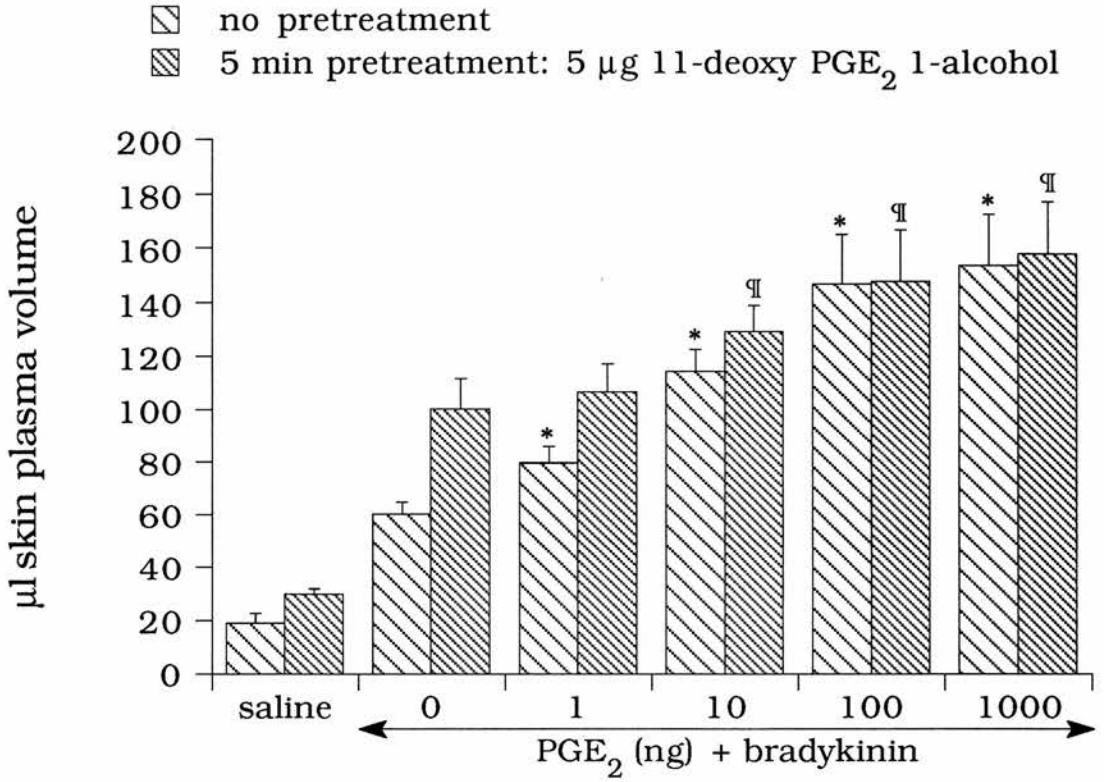


Figure 2.8 Effect of 11-deoxy PGE_2 1-alcohol (5 min pretreatment with 5 µg) on a system potentiated by PGE_2 in combination with 0.5 µg bradykinin (n=4).

* denotes significant difference from bradykinin response alone ($p<0.05$).
¶ denotes significant difference from bradykinin response following pretreatment with 11-deoxy PGE_2 1-alcohol ($p<0.05$).

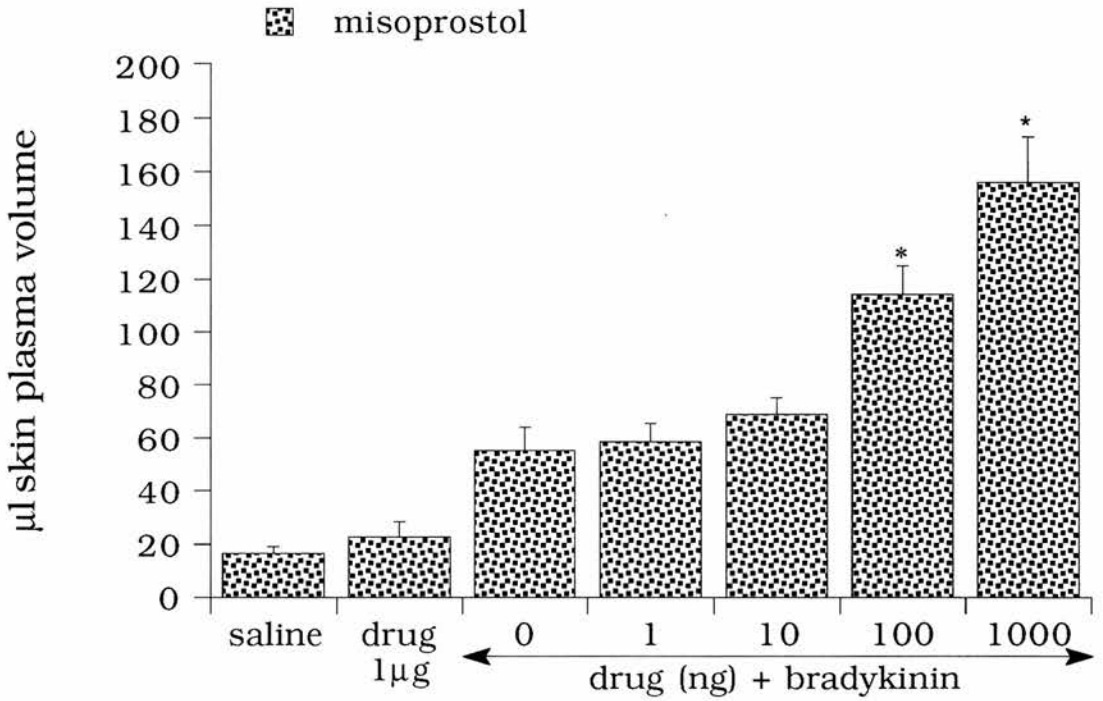


Figure 2.9 Potentiation of 0.5 µg bradykinin-induced plasma exudation by misoprostol (n=4).

* denotes significant difference from bradykinin response alone ($p<0.05$)

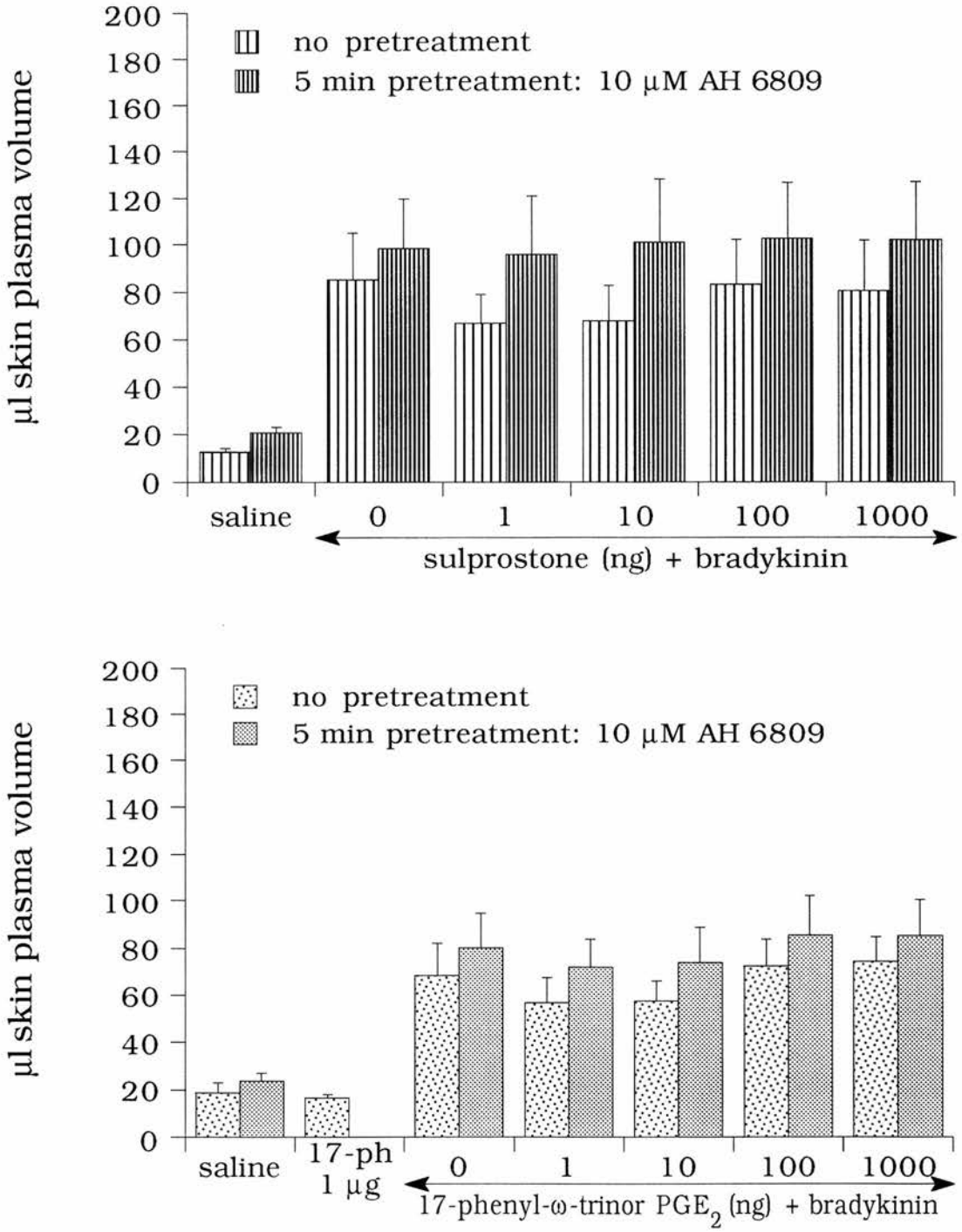


Figure 2.10 Effect of sulprostone (n=3) and 17-phenyl-ω-trinor PGE_2 (n=4) on plasma exudation induced by 0.5 µg bradykinin, and the influence of AH 6809 (10 µM, 5 min) thereon.

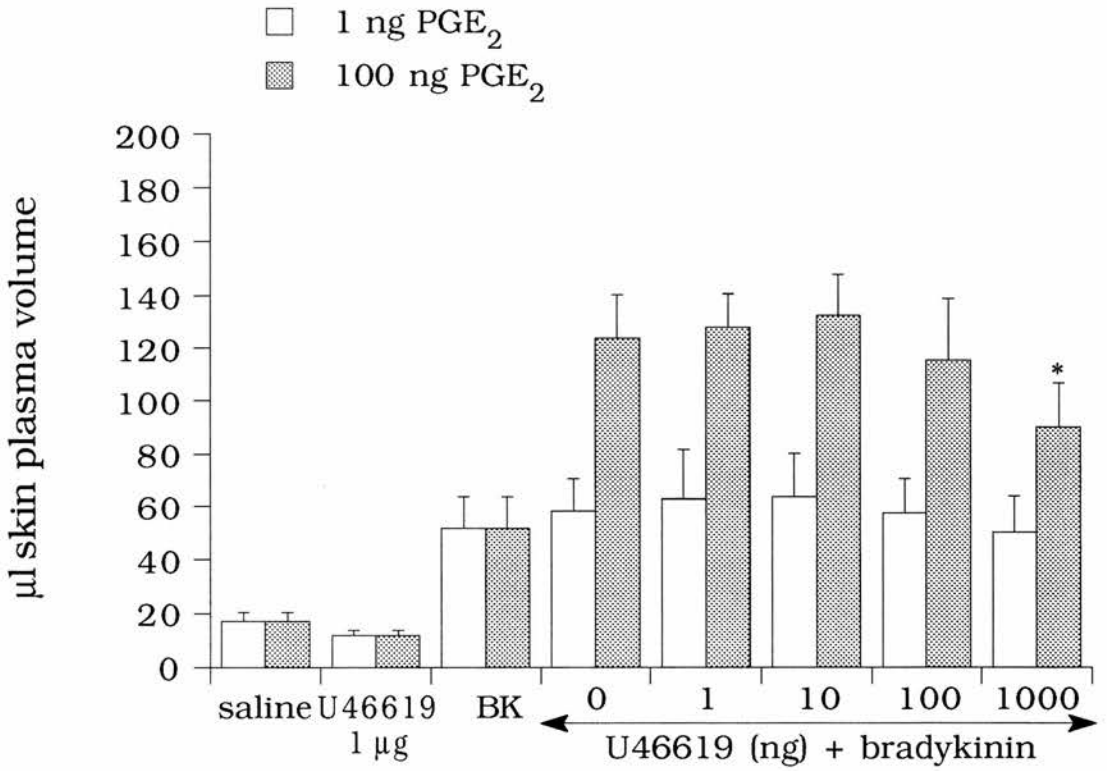


Figure 2.11 Dose-response to U46619 (n=4) on 1ng and 100 ng PGE_2-induced potentiation of plasma exudation to 0.5 µg bradykinin.

* denotes significant difference from PGE_2-induced potentiation of bradykinin response ($p<0.05$).

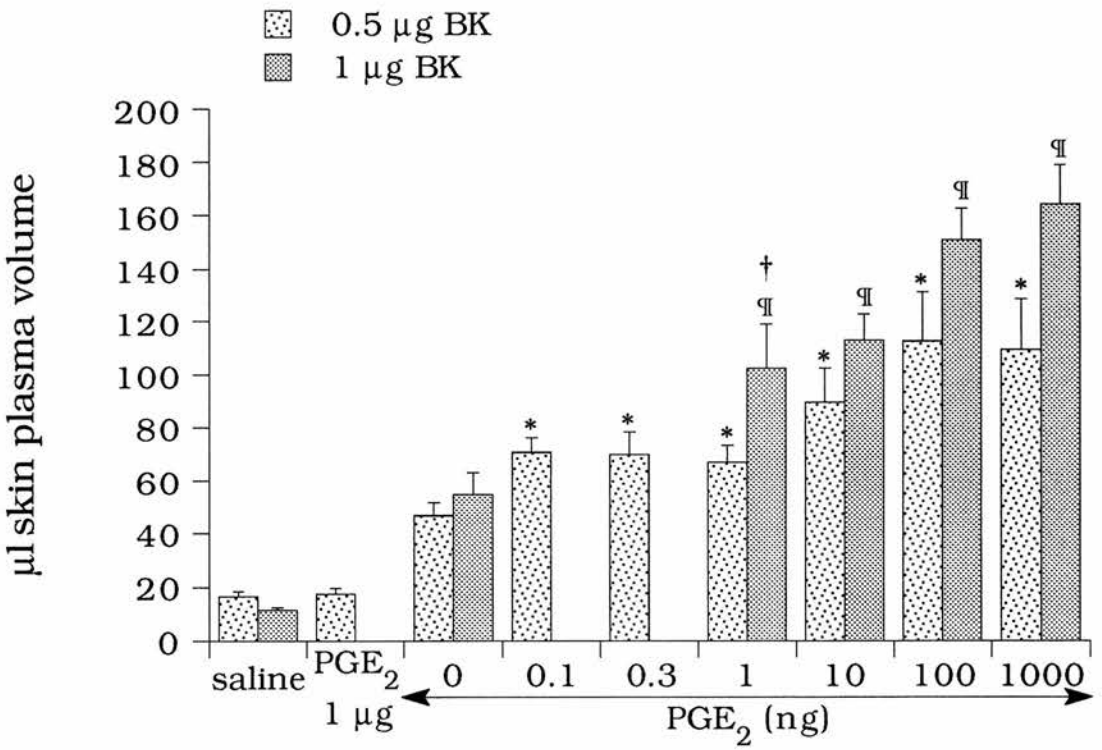


Figure 2.12 A comparison of the potentiation by PGE_2 of plasma exudation induced by 0.5 µg (n=4-12) and 1 µg (n=4) bradykinin.

* denotes significant difference from 0.5 µg bradykinin response alone ($p<0.05$).
¶ denotes significant difference from 1 µg bradykinin response alone ($p<0.05$).
† denotes significant difference from 1 ng PGE_2 + 0.5 µg bradykinin ($p<0.05$).

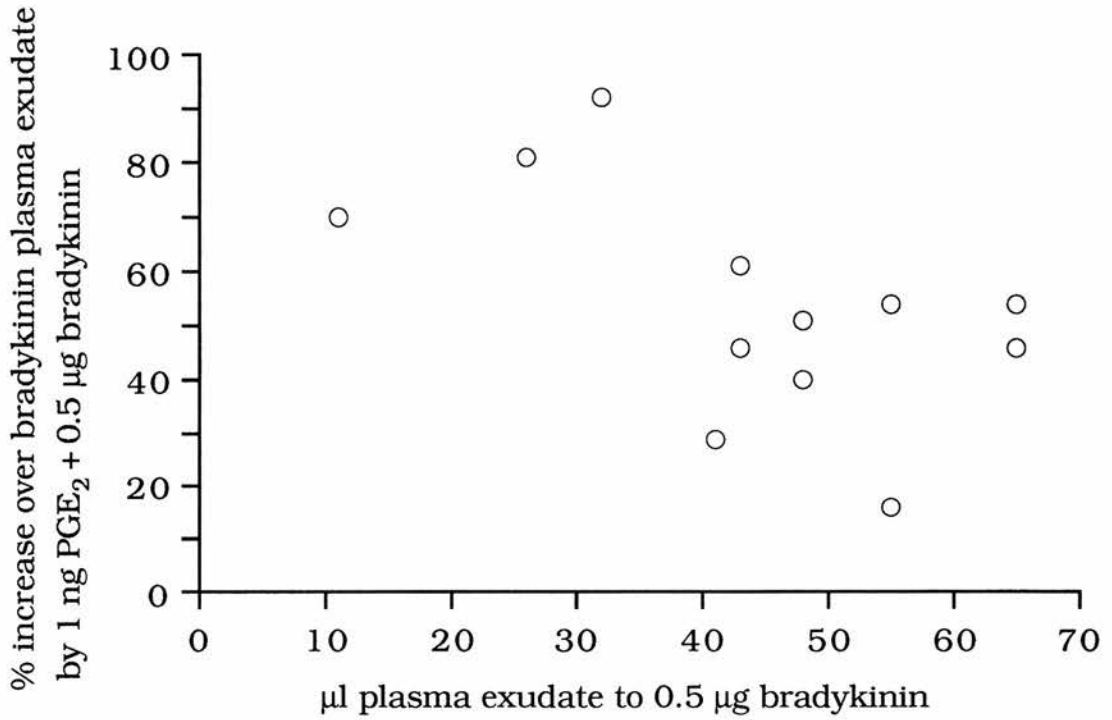


Figure 2.13 % increase over bradykinin response by 1 ng PGE_2 and 0.5 µg bradykinin in combination versus the bradykinin response alone expressed as µl plasma exudate.

2.4 DISCUSSION

In this *in vivo* rabbit model of skin inflammation, locally injected prostaglandins, whilst alone having no significant effect on vascular permeability, potentiated the plasma exudation induced by inflammatory mediators. The time-course of plasma exudation (Figure 2.1) indicated that a time-period of 30 min provided appreciable but submaximal response to both 0.5 μg bradykinin and 100 ng PGE_2 + 0.5 μg bradykinin. This is necessary in assessing potentiation or inhibition of such responses by additional agents. The slight plasma exudation induced by PGE_2 (Table 2.1) and the bradykinin responses listed (Table 2.2), are comparable to those reported elsewhere (for example Williams & Peck, 1977), where PGE_2 (300 ng) and bradykinin (300 ng) gave 8 μl and 40 μl skin plasma exudate respectively. PGE_2 has been shown to significantly potentiate the response to 0.5 μg bradykinin (Figure 2.3). Approximately one third of the maximum bradykinin-potentiating response to PGE_2 was achieved at 1 ng.

The effects of butaprost and 11-deoxy PGE_1, which are selective EP_2-receptor agonists mediating vasodilatation through increases in cyclic AMP, predicted the involvement of this receptor subtype. The lower potency of butaprost, as compared to PGE_2, in potentiating the response to bradykinin was surprising (Figure 2.6), since vasodilatation was reported to be responsible for the potentiating effect of PGE_2, and is typically mediated via the EP_2-receptor subtype. Whilst the actual skin plasma volume approached the maximium achieved with PGE_2, the % increase over bradykinin alone was only slightly greater than half. Preliminary experiments with 11-deoxy PGE_1, showed it to be active in potentiating the response to bradykinin. Whilst butaprost produced no significant increase until the 1 μg dose, 11-deoxy PGE_1 appeared to dose-dependently potentiate the response to bradykinin, a third of the maximium response being achieved at 50 ng.

11-deoxy PGE_2 1-alcohol, another EP_2-selective agonist, was less potent than butaprost (Figure 2.6). 0.1 μg - 5 μg 11-Deoxy PGE_2 1-alcohol produced only small increases of plasma exudation and only at 0.5 μg was the effect statistically significant. A similar % increase over bradykinin control was achieved by 1 ng PGE_2. It may be that 11-deoxy PGE_2 1-alcohol is behaving as a partial agonist. In order to test this theory, the effect of a 5 min pretreatment with 5 μg 11-deoxy PGE_2 1-alcohol on the ability of PGE_2 to potentiate the response to 0.5 μg bradykinin was investigated (Figure 2.8).

Whilst in the absence of 11-deoxy PGE_2 1-alcohol, PGE_2 was able to significantly potentiate the response to bradykinin over the range 1 ng to 1 μg doses, following 11-deoxy PGE_2 1-alcohol pretreatment the potentiation at 1 ng no longer achieved significance. This may reflect a partial agonist effect on low dose PGE_2. The analysis of such a study is complicated by the observation that following pretreatment, the bradykinin control was significantly increased ($p<0.05$). This may be the result of double injections into sites receiving pretreatment, and with hindsight, may have been accounted for by injecting saline into those sites not receiving pretreatment.

The increase induced by 11-deoxy PGE_2 1-alcohol pretreatment is similar to an effect of 3 ng PGE_2 + bradykinin, as estimated from the dose-response relationship to PGE_2 shown in Figure 2.8. Assuming simple addition of effects, 5 μg 11-deoxy PGE_2 1-alcohol in combination with 3 ng PGE_2 + bradykinin should equal 4 ng PGE_2 + bradykinin, which it does. Therefore, since there is no antagonism between 11-deoxy PGE_2 1-alcohol and PGE_2 here, the former is not behaving as a partial agonist in this system. As a control, 10 μg PGE_2 + bradykinin was included and gave similar responses both in the absence and presence of 11-deoxy PGE_2 1-alcohol pretreatment.

The EP_2- and EP_3- selective agonist misoprostol, in combination with bradykinin, was the only analogue to produce a maximum response (194 %) comparable to that for PGE_2 (167 %) (Figure 2.9). However, whilst at 1 ng, PGE_2 significantly potentiated the response to bradykinin, significance was not achieved with misoprostol until the 100 ng dose. The possibility of EP_3-receptor involvement was addressed using sulprostone, an EP_1- and EP_3- receptor agonist. In an effort to dissociate action at these two receptor subtypes, studies with sulprostone were carried out in the presence and absence of a 5 min pretreatment with the EP_1-receptor antagonist, AH 6809. Sulprostone alone did not significantly increase the response to bradykinin at doses up to 1 μg. There did not appear to be any inhibitory influence of an EP_1-receptor system on an EP_3- mediated effect, since AH 6809 did not significantly alter the % increase over control, as calculated relative to the bradykinin response following AH 6809 pretreatment. This was considered the best analysis for significance, since AH 6809 pretreatment itself significantly increased the bradykinin response, as discussed previously for 11-deoxy PGE_2 1-alcohol pretreatment.

In order to study the effect of an EP_1-selective agonist on the bradykinin response, 17-phenyl-ω-trinor PGE_2 was tested in this model (Figure 2.10). A similar profile to sulprostone was observed, in that there was no potentiation of the response to bradykinin, and AH 6809 pretreatment did not significantly alter these results. It was reasoned that a possible EP_1-mediated inhibitory effect may only be evident on a potentiated system. Preliminary studies have therefore been carried out using 100 ng PGE_2 + 0.5 μg bradykinin to potentiate the response to bradykinin, pretreatment with AH 6809 being used to determine its influence on any 17-phenyl-ω-trinor PGE_2 effect. At 1 μg 17-phenyl-ω-trinor PGE_2, there was a slight reduction in the response to PGE_2 + bradykinin, an effect which appeared to be blocked by AH 6809 pretreatment. In order to assign significance to this slight EP_1-mediated inhibitory effect, further studies are required.

Some PGE analogues such as MB 28767, whilst being selective for the PGE subtypes, also have agonist activity at the TP receptor. The TP-receptor agonist U46619 at a dose of 1 μg, significantly inhibited the exudation response to 100 ng PGE_2 + bradykinin, and reduced that to 1 ng PGE_2 + bradykinin (Figure 2.11). The small increase over bradykinin-induced plasma exudation by 1 ng PGE_2 may have prevented detection of any significant reduction by U46619 in this system. Nonetheless, this inhibitory influence via the TP-receptor raises the possibility that the full potentiating response to MB 28767 is not seen.

Since the bradykinin exudation responses vary between experiments, the influence of this variation on the potentiation observed by PGE_2 or its analogues, was of concern. A comparison of the potentiation of the response to both 0.5 μg and 1 μg bradykinin (Figure 2.12), have shown significant differences to occur only at the 1 ng dose. Since these were unpaired experiments, 100 ng PGE_2 + 0.5 μg bradykinin was included as a control in the 1 μg bradykinin studies. This was in fact found to be significantly different from 100 ng PGE_2 + 1 μg bradykinin.

The responses to 0.5 μg and 1 μg bradykinin in these studies were not significantly different from each other, and certainly do not represent the span over which the responses to bradykinin have been found to vary. Perhaps then, a greater fold difference in doses of bradykinin would be more representative, or indeed a dose-response to bradykinin in the presence of one submaximal dose of PGE_2. Ideally a dose-response curve to bradykinin in advance of test agents would allow the choosing of a bradykinin dose producing a similar effect in each experiment, but this is obviously not possible. Even incorporating a dose-response curve to bradykinin into a study to determine where the response lies in relation to the maximum, would only serve to limit the number of test injection sites in the study. Such differences in control response may be the result of variations in temperature or indeed seasonal variations in the rabbits themselves. Indeed, the absence of TP receptors from rabbit aorta over a six month period, has previously been reported (Tymkewycz *et al.*, 1991).

An alternative to looking at potentiation of an increased bradykinin dose, is to determine the relationship between response magnitude to bradykinin and the potentiation thereof. This is of importance since response magnitude may be more relevant than bradykinin dose. A graph was plotted of % increase over bradykinin response by 1 ng PGE_2 and 0.5 μg bradykinin in combination, against bradykinin response expressed as μl plasma exudate (Figure 2.13). Analysis of the data points showed no significant correlation suggesting the degree of potentiation to be independent of the bradykinin response alone, over the range observed for this dose of bradykinin.

The potentiation induced by PGE_2 has been suggested to result from its vasodilator activity (Williams & Morley, 1973). Previous studies have investigated this by comparing the ability of PGE_2 with that of PGI_2, to both increase blood flow and potentiate plasma exudation in the model of rabbit skin inflammation (Peck & Williams, 1978; Williams, 1979). PGE_2 and PGI_2 were potent at increasing blood flow, and showed a similar potency in potentiating the response to both bradykinin and zymosan. However, PGI_2 is unstable with a $t_{1/2}$ at 37 °C of ~ 3 min (Dusting *et al.*, 1977). Consequently I have compared the action of PGE_2 with that of a stable PGI_2 analogue, cicaprost, which has considerable specificity for the IP receptor. Similar increases in blood flow were demonstrated with cicaprost as compared to those reported for PGI_2 (Williams, 1979), though whilst this paper reports PGE_2 to be 10-fold more potent than PGI_2, I have found it to be essentially equiactive with cicaprost. The results reported here for the potentiation of the bradykinin response are in agreement with those reported by Williams (1979) for that to PGE_2 and PGI_2. It is interesting that in the paper by Peck & Williams (1978), whilst the increases in blood flow are reportedly much greater for both PGE_2 and PGI_2 than that reported by Williams (1979), there is no correspondingly greater increase in the volumes of exudate.

Here the maximum increase in blood flow in response to 0.1 ng or 1 μg PGE_2 was obtained following a 20 min time-period

(Figure 2.2). This is taken to be representative of the vasodilating capacity for the doses of PGE_2 tested, and subsequent measurements are taken following this regimen, when assessing the contribution of a vasodilator component to the potentiation of exudation by other inflammatory modulators. Cicaprost was shown to dose-dependently potentiate the response to bradykinin (Figure 2.3), the response being significantly different from that to PGE_2 only at the 1 ng dose, when more than 50 % of the maximal response to either was produced. Whilst significant increases over the bradykinin response were evident from 0.1 ng PGE_2 and 0.3 ng cicaprost, significant increases in blood flow were only demonstrated from 1 ng of either (Figure 2.4). In the absence of a significant increase in blood flow then, there was potentiation of the response to bradykinin by PGE_2 at 0.1 ng and 0.3 ng, and cicaprost at 0.3 ng. Perhaps vasodilatation itself is not wholly responsible for the potentiation of the bradykinin response by these two agents, and another common factor is responsible for their potentiating activity at the lower doses. Also, the significantly greater increases in blood flow induced by PGE_2 at 100 ng and 1 μg as compared to cicaprost, did not result in a significant difference in their responses to bradykinin. Indeed, whilst no significant increase was detected between the potentiation of bradykinin induced by PGE_2 as compared to cicaprost at 100 ng or 1 μg, it was cicaprost and not PGE_2 that produced the greater absolute increase in skin plasma exudate, and the greater % increase over the response to bradykinin alone.

The responses of PGE_2 and cicaprost in combination with the leucocyte-dependent chemoattractant, FMLP were only significantly different from that to FMLP alone at the lower doses, and there was no significant difference between the response of FMLP to either, at any of the doses tested. PGE_2 and cicaprost therefore, in the absence of a significant increase in blood flow, significantly potentiated the response to FMLP. FMLP has been reported to produce a small plasma exudation, which is not dependent on histamine release and may be potentiated by PGE_2 (Hellewell *et al.*, 1989). In potentiation of the FMLP response,

there does not appear to be a good correlation between the dose of PGE_2 or cicaprost, and the increase in μl skin plasma volume exudated at the lower concentrations. Full dose-response curves were not conducted in the same rabbits, the range 0.1 ng to 1 ng being carried out separately from 1 ng to 1 μg. The response to 1 ng of either PGE_2 or cicaprost was greater in the former, which were conducted (end July - beginning August) six months after those at the higher doses (end January - beginning February), highlighting the problem with seasonal and temperature variations already mentioned. The results at 1 ng were averaged and therefore lie between the higher response at 0.3 ng and the lower response at 10 ng for either PGE_2 or cicaprost.

11-Deoxy PGE_2 1-alcohol produced an increase in plasma exudation at 500 ng (Figure 2.6). This represented a significant increase over that to bradykinin alone, and occurred in the absence of any significant alteration in blood flow (Figure 2.7) highlighting the possible existence of another mechanism besides vasodilatation in the potentiation of inflammatory mediator action.

In summary, the effect of the drugs tested on this *in vivo* model of rabbit skin inflammation, suggests an EP_2-like receptor subtype to be involved in the potentiation of the inflammatory response induced by mediators such as bradykinin. There are discrepancies between the changes in blood flow and plasma exudation which suggest that whilst vasodilatation may be important in the potentiation response, it does not appear to be the sole mechanism by which the effect is mediated.

These studies have been complemented and furthered by the work of a colleague, which gives a greater insight into the mechanisms involved in PGE_2-induced potentiation. The EP_3-component of PGE_2 activity was additionally examined using the EP_3-selective analogue, MB 28767, following inhibition of its TP-receptor activity by GR 32191 (Armstrong *et al.*, 1990). At 1 ng MB 28767 significantly increased plasma exudation to +78 % but there was no further increase up to the maximum dose tested at 1 μg. This may reflect MB 28767 overcoming the TP-receptor block at the higher doses, agonist activity at this receptor exerting an inhibitory

influence on the potentiation as demonstrated by the experiments with the TX-mimetic, U46619 (Figure 2.11). However sulprostone, which is a very weak TP-receptor agonist, showed a similar effect. Misoprostol, like PGE_2, was shown to be a potent vasodilator, which may correlate with its ability to potentiate exudation (Figure 2.9). MB 28767 however, did not cause dilatation, though it was able to potentiate the response to bradykinin, as described.

The plasma exudation and dilatation results of the PGE analogues tested, taken in combination, suggest both EP_2- and EP_3- mediated events may be involved in this pro-inflammatory activity of PGE_2. The lack of activity of 17-phenyl-ω-trinor PGE_2 in potentiating the bradykinin response (Figure 2.10), suggests the EP_1-receptor is not involved, though the preliminary results on a potentiated system (see results section) may indicate an inhibitory component by this receptor subtype. It was concluded that the high potency component may reflect an EP_3-mediated activity, which would appear to be unrelated to vasodilatation. The potentiation of bradykinin exudation seen with agonists having EP_3-activity, may therefore result from bradykinin-induced dilatation potentiating an EP_3-induced neutrophil-dependent exudation, as shown for LTB_4 (Bray *et al.*, 1981).

As reported earlier, the increase in plasma exudation by 1 ng PGE_2 was significantly greater in combination with 1 μg bradykinin as compared to 0.5 μg. This may reflect a greater dilatation at 1 μg bradykinin increasing the response of an EP_3 component of potentiation, as represented by low concentrations of PGE_2. The lower potency component is proposed to be an EP_2-mediated vasodilatation, which may differ from that previously characterised by the high potency of butaprost. This would explain the observation that of all the analogues tested, misoprostol, having both EP_2- and EP_3- receptor activity, produced a similar maximum to that obtained with PGE_2 in potentiating the response to bradykinin.

Cicaprost, a potent vasodilator and PGI_2 analogue, has been shown

to potentiate plasma exudation induced by the EP_3-selective analogues MB 28767 and sulprostone, but not the EP_2-selective analogues butaprost and 11-deoxy PGE_1, in the absence of other inflammatory mediators (Armstrong & Jones, 1991). In order to determine if the EP_3-mediated effect is neutrophil-dependent, experiments were carried out on rabbits rendered leucopoenic with nitrogen mustard. Following such pretreatment, the potentiation by cicaprost of MB 28767 and sulprostone was prevented, whilst the potentiation of the bradykinin response was unaltered. This supports the proposition that PGE_2 may exert part of its pro-inflammatory action via an EP_3 receptor-mediated, leucocyte-dependent mechanism.

A comparison of the effect of EP_2- and EP_3-selective agonists on a neutrophil -dependent (FMLP) and -independent (bradykinin) inflammatory mediator, gives further strength to the proposal that two different mechanisms are involved in the potentiation of plasma exudation by PGE_2 (Armstrong *et al.*, 1991). The response to FMLP was potentiated by the EP_2-selective agonists, 11-deoxy PGE_1, butaprost, mexiprostol and 11-deoxy PGE_2 1-alcohol, and correlated with their ability to increase local blood flow. The EP_3-selective agonists, MB 28767, sulprostone and GR 63799X, were unable to potentiate the response to FMLP, with neither the PGs nor FMLP able to increase local blood flow. This was in contrast to the potentiation observed with bradykinin, which itself was able to increase local blood flow.

PGE_2, PGE_1, 6-keto-PGE_1 and PGD_2, but not $PGF_{2\alpha}$, PGI_2 and its analogues, have all been shown to inhibit PMN superoxide production in response to FMLP (Gryglewski *et al.*, 1987). PGE_2 and 6-keto-PGE_1 may reduce the FMLP-induced response through occupation of the FMLP receptor. In addition PGD_2 and the PGEs are thought to have a common mechanism of action through interaction with different binding sites (Rossi & O'Flaherty, 1989). The mechanism of this action of the PGEs is suggested to be the result of an increase in cyclic AMP, which neither PGI_2 nor cicaprost are capable of inducing (Hecker *et al.*, 1990). Having shown the EP_3-mediated effect to be dependent on the

presence of circulating neutrophils, the effect of the PGE analogues on chemotaxis is now being investigated. Also, the subtypes of PGE receptor present on the neutrophil are being further characterised through both binding and second messenger studies.

Whilst vasodilatation is obviously an important component of the potentiation of inflammation induced by PGE_2, this study has suggested that it is not the sole mechanism of action of PGE_2. Indeed, subsequent studies have indicated the chemotactic nature of the EP_3-selective analogues on the neutrophil to be important. In a previous study, PGE_2 and PGI_2, both at a dose of 10 ng, were found to be equiactive in potentiating plasma exudation, yet PGE_2 was shown to be more active than PGI_2 in increasing blood flow (Williams, 1979). This is thought to reflect a different time-course of action for the PGs, with PGE_2 peaking before PGI_2, or alternatively, there is another component to their potentiating ability which is more active with PGI_2 than PGE_2. In addition to the vasodilatory component, it has also been suggested that PGE_2 may sensitise agonist receptors on vascular endothelial cells, or exert its effect through modulation of endothelial cell cyclic AMP levels (Williams & Morley, 1973). Binding and second messenger studies with cultured endothelial cells may therefore be the next step in further understanding the mechanisms of the pro-inflammatory action of PGE_2.

Chapter 3

Inhibition of cyclic AMP production in human platelets by PGE analogues

3.1 INTRODUCTION

3.1.1 Prostaglandins and platelet aggregation

In the early years of prostaglandin research, arachidonic acid was shown to induce platelet aggregation and this was accompanied by prostaglandin formation (Ingerman, 1973). NSAIDs were also shown to inhibit irreversible platelet aggregation and the production of PGE_2 and $PGF_{2\alpha}$ (Smith & Willis, 1971; Kocsis *et al.*, 1973). However, these prostaglandins could not themselves induce platelet aggregation nor surmount the inhibitory effect of aspirin, though pre-incubation with arachidonic acid prevented the inhibitory effect of aspirin on collagen-, adrenaline- and ADP-induced platelet aggregation (Leonardi *et al.*, 1972). Therefore the existence of a potent inducer of platelet aggregation was proposed, whose enzymatic generation from arachidonic acid is suppressed by NSAIDs (Willis & Kuhn, 1973), but whose nature is distinct from PGE_2 and $PGF_{2\alpha}$ (Vargaftig & Zirinis, 1973). Subsequently, the isolation and structure of two prostaglandin endoperoxides, PGG_2 and PGH_2, formed during prostaglandin biosynthesis was reported. These agents induced aggregation of human platelets (Hamberg *et al.*, 1974).

However, it soon became apparent that further metabolism to an even more potent aggregator was possible (see Figure 1.1). The product TXA_2 has a unique strained acetal structure which readily hydrolyses under neutral conditions ($t_{1/2}$ ~ 30 s, 37 °C) to give the inactive TXB_2 (Hamberg *et al.*, 1975). A microsomal thromboxane synthetase system was subsequently identified and characterised from both human and horse platelets (Needleman *et al.*, 1976). This enzyme was able to convert the prostaglandin endoperoxides into TXA_2 which proved to be more potent in contracting strips of rabbit aorta. It was not long after that another enzyme was isolated, this time in microsomes prepared from rabbit or pig aorta. Interestingly this enzyme converted the prostaglandin endoperoxides into yet another unstable substance, PGI_2

(see Figure 1.1), originally termed PGX, (Johnson *et al.*, 1976), with opposing biological activities to TXA_2 (Moncada *et al.*, 1976).

3.1.2 Inhibition of platelet aggregation - who shares what receptor?

PGE_2 and PGE_1 have both been shown to raise cyclic AMP levels and inhibit ADP-induced platelet aggregation, though PGE_1 was the more potent of the two (McDonald & Stuart, 1974). PGI_2 however, was found to be much more potent than PGE_1 in inhibiting platelet aggregation, and is in fact the most potent inhibitor of human platelet aggregation *in vitro* so far described (Moncada *et al.*, 1976). It also is a potent stimulator of cyclic AMP accumulation in human platelet-rich plasma and a direct stimulator of platelet microsome adenylate cyclase, with an e.m.r. of 0.1 relative to PGE_1 (Gorman *et al.*, 1977).

Following the report that PGD_2 could also elevate platelet cyclic AMP levels (Tateson *et al.*, 1977), the possibility of a common receptor was addressed. The sensitivity of PRP from horse, human, rabbit, rat and sheep to PGE_1 followed more closely the sensitivity to PGI_2 than to PGD_2, suggesting there may be a common site for PGE_1 and PGI_2 (Moncada *et al.*, 1977). However, distinct receptors were believed to exist on the platelet for PGI_2 and PGD_2 (Siegl *et al.*, 1979a,b; Siegl, 1982). This was supported by binding studies on normal citrated human PRP, which revealed PGE_1 to be 20-fold less potent than PGI_2 in displacing $[^3H]$-PGI_2, whilst PGD_2 was ineffective despite being more potent than PGE_1 as an inhibitor of ADP-induced aggregation *in vitro*. Also, di-4-phloretin phosphate blocked the inhibitory effect of PGD_2 but not PGE_1 or PGI_2 in human platelet aggregation (Westwick & Webb, 1978). The inhibition of irreversible, monophasic, ADP-induced aggregation by PGI_2 and PGD_2, was found to be additive (Andersen *et al.*, 1980), which is consistent with the presence of two structurally distinct receptors governing the same adenylate cyclase.

Further evidence for a common PGE_1 / PGI_2 receptor comes from

desensitisation studies to elevation of cyclic AMP in human PRP (Miller & Gorman, 1979). Whilst PGE_1 and PGI_2 reciprocally desensitise to subsequent challenge, PGD_2 desensitises platelets to later challenge by PGD_2 but not PGE_1 or PGI_2. This PGE_1 / PGI_2 receptor has since been purified from human blood platelets and binding of $[^3H]$-PGE_1 displaced by PGI_2 but not PGD_2 (Dutta-Roy & Sinha, 1987). PGE_1 and PGE_2 may also share a receptor on the human platelet having a similar potency in displacing $[^3H]$-PGE_1 from its high affinity binding sites in the platelet suspension (McDonald & Stuart, 1974), but not PGD_2 from its own receptor (Bonne *et al.*, 1981). This is distinct from the PGE_1 / PGI_2 receptor already discussed, since specific binding of $[^3H]$-PGE_2 to its site was displaced by PGE_2 and PGE_1, but not by PGI_2 (Eggerman *et al.*, 1986).

3.1.3 What is the role of PGE_2 in platelet aggregation ?

Kloeze's first report (1969) of the stimulatory activity of PGE_2 on ADP-induced platelet aggregation in the rat, in contrast to its inhibitory effect towards human platelets, prompted a reexamination of PGE_2 activity by Shio and Ramwell (1972). They, and others since, found PGE_2 to be distinct from other prostaglandins in that at low concentrations it potentiates human platelet aggregation induced by ADP or arachidonic acid, yet at higher concentrations platelet inhibition is observed (Shio & Ramwell, 1972; Vargaftig & Chignard, 1975).
MacIntyre & Gordon (1975) found PGE_2 to reproducibly enhance aggregation in heparinised PRP from both rat and man, and indeed to directly induce aggregation in pig heparinised PRP. PGE_2 often shows a dual effect on aggregation, inhibiting primary aggregation and stimulating release (^{14}C-5HT loading technique) in secondary irreversible aggregation (Shio & Ramwell, 1972; McDonald & Stuart, 1974; Andersen *et al.*, 1980).
Andersen *et al.* (1980) have suggested the aggregation-potentiating effect of PGE_2 occurs at concentrations as low as 10-20 nM, and is therefore of physiological significance.

The pro-aggregatory effect of PGE_2 is reported to be responsible in part for the lack of sensitivity of platelet aggregation to five different thromboxane synthetase inhibitors in non-responders (Gresele *et al.*, 1988). Inhibition of thromboxane synthesis results in the accumulation of the PG endoperoxides which may be metabolised further to PGD_2, PGE_2 and $PGF_{2\alpha}$, or under the influence of endothelial PGI_2 synthetase be converted into PGI_2. There is also the potential for the PG endoperoxides themselves to stimulate platelet aggregation, through interaction with the TP-receptor.

In vitro, inhibition of arachidonic acid - induced platelet aggregation in responders due to the presence of such inhibitors was observed, and PGE_2 was mostly anti-aggregatory. However, in those cases where the thromboxane synthetase inhibitors did not inhibit the arachidonic acid - induced platelet aggregation, PGE_2 was found to be pro-aggregatory at similar concentrations of 50 - 500 nM. Neutralisation of endogenously produced PGE_2 by an anti-PGE_2 serum, transformed nonresponders into responders, indicating the involvement of the pro-aggregatory PGE_2 in the arachidonic acid - induced aggregation response, obtained in the presence of the thromboxane-synthetase inhibitors in non-responders. The response of the platelets is thought to be dependent on the balance between PGD_2 and PGE_2, since if PGD_2 formation is enhanced by the addition of serum albumin, non-responders to the TX synthetase inhibitor dazoxiben are induced to respond (Gresele *et al.*, 1984).

The pool of platelets were equally sensitive to the effects of forskolin or dibutyryl cyclic AMP, suggesting no change in the sensitivity of the catalytic unit between responders and non-responders. Further, in the absence of any specific receptor effect, the difference in response to the thromboxane synthetase inhibitors is thought to be at the level of coupling between the EP-receptor and the adenylate cyclase unit. With the additional presence of a TP-receptor antagonist there were no non-responders, suggesting the action of the accumulated PG endoperoxides in response to the inhibition of thromboxane

synthetase are responsible for the altered effects to PGE_2 and PGI_2.

3.1.4 Regulation of adenylate cyclase

Early reports have suggested that the potentiation of aggregation by low concentrations of PGE_2 is due to a decrease in cyclic AMP content of the platelet, with an increase at the higher concentrations inhibiting aggregation (Salzman *et al.*, 1972). Thus Gray and Heptinstall (1985) demonstrated potentiation of ADP- and collagen- induced aggregation by 10^{-8} - 10^{-6} M PGE_2 and inhibition at 10^{-5} M. Interestingly with adrenaline-induced aggregation, only the inhibitory component of PGE_2 was present. However, PGE_2 could also reverse the inhibitory effect of PGI_2 on adrenaline-induced aggregation. Since adrenaline itself inhibits adenylate cyclase in human platelets (Zieve & Greenough, 1969; Salzman & Levine, 1971), this may possibly prevent further depression by PGE_2 and therefore potentiation of aggregation was not demonstrated. PGI_2 inhibited the adrenaline-induced aggregation through elevation of cyclic AMP (Gorman *et al.*, 1977), thereby permitting the reversing action of PGE_2 through subsequent inhibition of cyclic AMP formation.

As PGE_1 acts on the IP receptor as already discussed, by analogy the reversal of the inhibitory effect of PGI_2 on adrenaline-induce aggregation by PGE_2 may reflect a degree of competition with PGI_2 for the inhibitory IP receptor, at which PGE_2 is the weaker agonist (Miller & Gorman, 1979). However, PGE_2 is an effective antagonist to PGE_1, PGD_2 and PGI_2 (Andersen *et al.*, 1980; Bonne *et al.*, 1981) and inhibits the increase in cyclic AMP induced by adenosine and sodium fluoride (Bonne *et al.*, 1981), suggesting a separate receptor for the potentiation of aggregation by PGE_2.

The prostaglandins PGI_2 and PGD_2 have been shown to produce a time- and dose- dependent inhibition of their own rapid activation of adenylate cyclase in platelet lysates (Ashby, 1986) similar to previous reports of PGE_1 in intact platelets (Mills & Smith, 1972).

This may indicate the presence of a low affinity inhibitory PG receptor and subsequently a two receptor model was proposed to explain prostaglandin regulation of adenylate cyclase (Ashby, 1988,1989).

The fall in cyclic AMP level was shown to be independent of phosphodiesterase (PDE) activity. Platelet PDE may be activated by phosphorylation due to the action of cyclic AMP - dependent protein kinase (Grant *et al.*, 1988; MacPhee *et al.*, 1988). Such activation of PDE is however rapid (Alvarez *et al.*, 1981) and in the presence of a PDE inhibitor there is still a time-dependent fall in the cyclic AMP level.

By following the time-course of cyclic AMP metabolism in the intact cell, Ashby (1990) suggested distinct stimulatory and slow-acting inhibitory receptors differing in affinity for each of the prostaglandins. This proposal is supported by binding studies reporting two classes of prostaglandin binding site on platelet membranes (Schafer *et al.*, 1979; Siegl *et al.*, 1979a,b; Siegl, 1982). The model that Ashby proposes does not require that any of the prostaglandins binds to the same receptor, the desensitisation which he refers to being at the level of the G proteins, reflecting more a physiological antagonism.

3.1.5 Physiological role of the prostanoids

It has been proposed that the arachidonic acid metabolites have a role in the regulation of platelet interactions with the blood vessels (MacIntyre, 1979). Prostaglandins not normally produced by the platelets, such as PGE_1 and PGI_2, modify platelet function when added exogenously or formed endogenously by the lungs or vasculature, and released into the circulation. Studies conducted by Kloeze (1969) on platelet-rich rat blood plasma, indicated that PGE_1, which we now know to be acting through the IP-receptor, inhibited the ADP-induced platelet aggregation through a rise in cyclic AMP. The relationship of the platelet with the blood vessel is such that in the case of a normal undamaged vessel, the endothelium plays an active part in the prevention of thrombus

formation. Part of this may be due to the generation and release of PGI_2 to balance the opposing action of released prostaglandin endoperoxides which stimulate platelet aggregation (Gryglewski *et al.*, 1976). The endothelial cells may form this PGI_2 utilising PGH_2 released from activated platelets in close association with the vascular endothelium (Bunting *et al.*, 1976). Platelet aggregation *in vivo* is thought to be regulated in part by a balance between platelet TXA_2 and endothelial PGI_2.

The main physiological role of platelets is the triggering of haemostasis where they are important for healing damaged blood vessels (Mustard & Packham, 1970). If a vessel is cut or injured the endothelium will be damaged and the subendothelial tissue exposed. As a result of their strong affinity for collagen, platelets adhere to the exposed subendothelial tissue in the damaged area and become activated. The release reaction following shape change and aggregation will recruit more circulating platelets and a plug will form as a consequence. Along with vessel constriction, this plug maintains haemostasis in the small vessels until the platelet plug is reinforced by fibrin. This sequence of events may be inappropriately triggered in a number of pathophysiological conditions including tumour cell metastasis, asthma, migraine, atherosclerosis, and most prominently, thrombosis. A bleeding disorder will result if platelets cannot aggregate or if the platelet count is low, as in thromocytopenic purpura. It is in these circumstances where drugs which influence the aggregation process may be of therapeutic value.

3.1.6 The mechanism of platelet aggregation

The response of a stirred suspension of platelets to an activating agent progresses from shape change, through aggregation, to release of granule contents, and occurs in response to a variety of different agonists (reviewed in Seiss, 1989). Quantitative studies of shape change and aggregation have been facilitated by the invention of the platelet aggregometer by Born in 1962. Light passing through the platelet suspension is detected by a photocell

connected to a chart recorder. Shape change results in a small, rapid reduction in light transmission and a disappearance of oscillations as the platelets become spherical and extrude pseudopodia. The disc shape characteristic of the resting state of the platelet is maintained by a ring of microtubules around the edge below the plasma membrane. The rounding of the platelet is associated with constriction of the ring of microtubules and centralisation of the organelles within the ring. Cytoplasmic actin- and myosin- like proteins also cross-react. Shape change is not a prerequisite for aggregation, as adrenaline can cause aggregation without shape change and cytochalasin B inhibits shape change but not aggregation. When aggregation occurs there is an increase in light transmission dependent on extracellular Ca^{2+} and fibrinogen, which forms bridges between platelets, and only occurs if the platelet suspension is stirred rapidly enough (1000 rpm) to allow the platelets to collide. The rate and extent of the increase is dependent on the concentration of the aggregatory agent. Fibrinogen binding to activated platelets and the subsequent cross-linking gives rise to the reversible primary agregation obtained with weak aggregatory agents, or a low concentration of the stronger agonists. Stronger stimuli give rise to secondary irreversible aggregation associated with prostaglandin endoperoxide or thromboxane synthesis, and release of granule contents.

Platelets contain three types of storage granules whose contents are released upon activation. Dense granules containing 5HT, ADP, ATP, Ca^{2+} and Mg^{2+}, influence vascular tone and the thrombus-forming ability of other platelets. α-Granules contain proteins which influence blood vessel function and the clotting cascade, such as platelet factor 4, β-thromboglobulin, platelet-derived growth factor (PDGF), thrombospondin and fibrinogen. PDGF is secreted by platelets when serum is formed from plasma and gives serum its property of stimulating or sustaining growth of cells in culture. The contents of the lysosomal granules are only released after platelets are stimulated with powerful aggregating agents such as thrombin and high

concentrations of collagen. The irreversibility of the secondary aggregation may be due to the stabilisation of the fibrinogen bridges by thrombospondin, the adhesive protein released from the α-granules.

Besides the contents of the granules, activated platelets can release physiologically active substances, which are newly synthesised rather than stored. Platelet activating factor (PAF-acether, PAF) is generated from most inflammatory cells when they are activated, and from platelets by the action of thrombin. PAF produces many of the phenomena of inflammation including induction of local platelet aggregation (Chignard *et al.*, 1979). The effects are produced at low concentrations (1-10 nM) and though human platelets do not produce detectable amounts on physiological activation, PAF is important in bronchial hyperresponsiveness, the delayed phase of asthma and may also have a role in arterial thrombosis and endotoxic shock (Braquet *et al.*, 1987). Since aspirin inhibits the second wave of aggregation, first shown by O'Brien (1968), the endoperoxides have been implicated in the release reaction (Samuelsson *et al.*, 1976). Generally it is the act of aggregation rather than the agonist itself which causes prostaglandin synthesis and release, as unstirred platelets or those prevented from aggregating by treatment with an antibody to glycoprotein IIb / IIIa, thought to be the fibrinogen receptor, do not release their granule contents, whereas centrifugation of activated platelets will cause release (Balduini, 1988). Thrombin is the exception, in that the resulting TXA_2 formation and release appear unrelated to aggregation. Other substances released from platelets are factors which increase vascular permeability, factors chemotactic for white blood cells and platelet-derived growth factors which can cause proliferation of fibroblasts, vascular endothelial cells and vascular smooth muscle cells.

There are several forms of positive feedback control in the aggregation of platelets. Activation may be enhanced due to the release of ADP from the dense granules. Following induction of arachidonic acid metabolism, the stimulatory PGG_2, PGH_2 and TXA_2 further enhance aggregation and cause this release reaction

(Malmsten *et al.*, 1975). Indomethacin was shown to inhibit the PGG_2 formation and the release reaction induced by collagen, ADP, adrenaline and thrombin, but had no effect on the PGG_2-induced release reaction. The aggregating effect of PGG_2 was however abolished by furosemide, a competitive inhibitor of ADP-induced primary aggregation. The results suggest these aggregating agents require PGG_2 (and / or PGH_2) synthesis for induction of the release reaction, the resulting ADP promoting the aggregation. Strong stimuli such as collagen or thrombin may also induce the synthesis and release of PAF which, as discussed, causes platelet aggregation at a low concentration. All these agents are important in activating more platelets to increase the size and effectiveness of the thrombus. How this positive feedback is limited is not well understood. Dilution by the flow of blood past the thrombus may limit their effective concentration, and the PGI_2 released by stimulation of the vessel wall will be exerting opposing inhibitory effects on the platelets.

3.1.7 Second messengers

The most important stimuli for platelet aggregation *in vivo* include collagen fibres to which the platelet is exposed following vascular damage, ADP released from damaged cells and thrombin which is formed rapidly from circulating prothrombin at the sites of platelet adherence. Stimulatory agonists including collagen, thrombin, PAF, vasopressin and TXA_2, activate phospholipase C (PLC), which hydrolyses phosphatidyl-inositol 4,5-bisphosphate (PIP_2) (Agranoff *et al.*, 1983) producing the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG) (see Figure 3.1.1). IP_3 causes Ca^{2+} release from the dense tubules and DAG activates protein kinase C (PKC) (Drummond & MacIntyre, 1987). However, subsequent action of DAG kinase may result in the formation of phosphatidic acid (PA), from which free arachidonic acid may be released by the action of a PA-specific PLA_2 (Billah *et al.*, 1981). PA may additionally be produced as a result of the PLD-induced hydrolysis of PC, as demonstrated for thrombin in

human washed platelets (Rubin, 1988). Alternatively, sequential action of DAG lipase and monoacylglycerol lipase may directly produce arachidonic acid (Bell *et al.*, 1979). The cyclooxygenase products of this arachidonic acid exert a positive feedback on the platelet aggregation as already described.

Adrenaline and ADP may activate PLC indirectly through formation of TXA_2, since cyclo-oxygenase inhibitors or TX receptor blockers strongly reduce the inositol phosphate production in response to these agonists (Sweatt *et al.*, 1986a). Multiple forms of PLC activity have been described for the soluble (Mauco *et al.*, 1979; Rittenhouse-Simmons, 1979; Billah *et al.*, 1980) and particulate (Banno *et al.*, 1988) extracts of human platelets.

The involvement of Ca^{2+} in platelet activation results from studies using the Ca^{2+} ionophore A23187, which mimics stimulatory agonists in causing shape change, aggregation and the release reaction (Feinman & Detwiler, 1974). Tsien *et al.* (1984) revolutionised the measurement of $[Ca^{2+}_i]$ and its role in stimulating response coupling, by the invention of a fluorescent Ca^{2+} indicator, quin-2, and its more sensitive successor fura-2. In their esterified form they enter the intact cell and following deesterification become trapped. Ca^{2+} studies have also progressed through the use of the photo-protein aequorin and measurement of $^{45}Ca^{2+}$ fluxes. An increase in $[Ca^{2+}]_i$ was thought to be the common activator for platelet shape change, aggregation and release, as shown for ADP using quin-2 loaded human platelets (Hallam & Rink, 1985). Influx of Ca^{2+} across the plasma membrane was thought to be the major contributor to this increase, since it was markedly reduced in the absence of extracellular Ca^{2+} or in the presence of the Ca^{2+} chelator, EGTA. The small increase under these conditions would be the result of a release from internal stores. The Ca^{2+} content of the organelles is 10 - 20 mM but much of this is reversibly bound to a calsequestrin-like molecule, so that the free $[Ca^{2+}]$ is several 100 μM. The entry of extracellular Ca^{2+} is regulated by the state of filling of the discharged intracellular store.

By measuring both quin-2 fluorescence and the optical density of

the suspension in the presence of the selective Ca^{2+} ionophore, ionomycin, the threshold $[Ca^{2+}]_i$ for shape change was found to be 300 nM, becoming maximal at 800 nM with aggregation occurring at 1 μM. However, maximal shape change using ADP as the stimulus occurred at $[Ca^{2+}]_i < 200$ nM, producing an aggregatory response at $[Ca^{2+}]_i < 1$ μM. This ability of ADP to induce both shape change and aggregation with a smaller increase in $[Ca^{2+}]_i$ than that required by the calcium ionophore, ionomycin, suggests additional excitatory mechanisms besides the increase in $[Ca^{2+}]_i$ are involved in the response to ADP. A similar effect had previously been described for thrombin and PAF, which were able to produce aggregation and secretion even when $[Ca^{2+}]_i$ was suppressed. It was suggested that the DAG formed as a result of PIP_2 hydrolysis may be responsible for this Ca^{2+}-independent activation (Rink *et al.*, 1983), 1-oleoyl-2-acetyl-glycerol (OAG) inducing secretion and aggregation without raising $[Ca^{2+}]_i$ above the basal concentration of 100 nM. OAG acts through the stimulation of PKC and indeed TPA, another activator of PKC, was also a potent stimulus for secretion and aggregation. An increase in $[Ca^{2+}]_i$ which itself was unable to produce an effect, enhanced the response to both OAG and TPA. Therefore, a combination of a rise in $[Ca^{2+}]_i$ induced by IP_3 and the action of DAG through the activation of PKC, together appear to be responsible for the response of the platelets to thrombin and PAF. The synergism between Ca^{2+} and DAG may explain why some agonists cause responses with smaller rises in $[Ca^{2+}]_i$ than expected using ionophores, as described for ADP.

Agents whose action is to induce or augment platelet aggregation may also be associated with a decrease in the platelet content of the cyclic nucleotide, cyclic AMP (see Figure 3.1.1). Various aggregatory hormonal factors such as adrenaline, ADP (Cooper & Rodbell, 1979), platelet-activating factor (Haslam & Vanderwel, 1982), vasopressin (Vanderwel *et al.*, 1983) and thrombin (Aktories & Jakobs, 1984) have been shown to inhibit adenylate cyclase in the platelet particulate fraction. Besides the PLC pathway and the inhibition of adenylate cyclase,

there is evidence that increased Na^+ / H^+ exchange may play a role in the activation of platelets by adrenaline and ADP (Horne *et al.*, 1981) via the sequential formation of TXA_2 and IP_3 (Sweatt *et al.*, 1986a,b). It has been proposed that elevation of pH_i, as a result of the effect of ADP on Na^+ / H^+ exchange, stimulates PLA_2 giving rise to TXA_2, and consequently IP_3 through activation of PLC. This IP_3 in turn mobilises Ca^{2+} from intracellular stores, resulting in the observed platelet aggregation. However, the ADP-induced dose-dependent Ca^{2+} mobilisation in fura-2 loaded platelets was not affected by indomethacin. It was however inhibited by > 65 % in indomethacin-treated platelets by the Na^+ / H^+ exchange inhibitor, ethylisopropylamiloride, an effect which could be overcome by artificially raising the pH_i. The effect of U46619, a stable TXA_2 mimetic, on Ca^{2+} mobilisation was similarly sensitive to EIPA. These results therefore show that Na^+/H^+ exchange is a common step in platelet activation by the PG endopeoxides / TXA_2 and ADP, and appears to enhance Ca^{2+} mobilisation independently of PLA_2 activity.

Inhibitory agonists act via elevation of intracellular cyclic AMP or cyclic GMP (Mills & Smith, 1971) (see Figure 3.1.2). Certain agents that elevate either cyclic AMP (PGE_1, PGI_2, forskolin) or cyclic GMP (organic nitrates, sodium nitroprusside, endothelium-derived relaxing factor (EDRF)) inhibit both vascular smooth muscle cells and platelet aggregation. The effect of a rise in cyclic AMP, accomplished either by inhibition of cyclic AMP phosphodiesterase or stimulation of adenylate cyclase, is to inhibit nonspecifically and noncompetitively platelet activation induced by aggregating agents, including Ca^{2+} ionophores, and to cause disaggregation if aggregation has already occurred (reviewed in Huang & Detwiler, 1986). It is of physiological significance that sodium nitroprusside and PGI_2 act synergistically to inhibit platelet aggregation (Levin *et al.*, 1982) since EDRF and PGI_2 are released together from the endothelium. EDRF is in fact nitric oxide, the effector molecule of the nitrovasodilators (reviewed in Moncada *et al.*, 1991).

The anti-platelet effect of nitrovasodilators was accompanied by a

large dose-dependent increase in cyclic GMP, and a smaller increase in cyclic AMP, which could both be blocked or reversed by the addition of the nitric oxide binding haemoglobin (Waldman & Murad, 1987). MB 22948, the selective inhibitor of cyclic GMP phosphodiesterase, potentiated the increase in both cyclic GMP and cyclic AMP caused by sodium nitroprusside. There is therefore an interaction between the guanylate and adenylate cyclase systems in the platelet, and it is suggested that cyclic GMP is inhibiting cyclic AMP phosphodiesterase by the phosphorylation of a cyclic GMP-dependent protein kinase (Maurice & Haslam, 1990). In effect cyclic AMP levels are elevated, thereby promoting the cyclic GMP-induced platelet inhibition.

The response of the platelet to stimulatory or inhibitory agonists is thought to be mediated largely by the phosphorylation of proteins (Rink & Hallam, 1984; Cohen, 1988; Rink & Sage, 1990). Aggregation can still occur, though it is much reduced, in the presence of non-specific inhibitors of protein kinase. This implies there is also a phosphorylation-independent pathway of platelet activation mediated by Ca^{2+} (Watson & Hambleton, 1989). Inhibitory agonists exert their effect by stimulating a specific pattern of protein phosphorylation in human platelets mediated by cyclic AMP- and cyclic GMP- dependent protein kinases (Waldman *et al.*, 1987). The activation of cyclic AMP - dependent protein kinase results in the phosphorylation of a protein which increases Ca^{2+} uptake, representing the major mechanism of inhibiting platelet function. This protein kinase may also inhibit PIP_2 hydrolysis, thereby inhibiting Ca^{2+} release as a result of IP_3 formation. Nitroprusside, through the increase of cyclic GMP levels, is a more effective inhibitor of platelet responses to different agonists rather than Ca^{2+} ionophores or phorbol ester, suggesting its main inhibitory action is at the generation of stimulatory second messengers.

The signal emitted from the activated receptor is transduced through the plasma membrane by GTP-binding proteins

(Gilman, 1987; Smith *et al.*, 1987). G-proteins are composed of three subunits α, β and γ and are associated with the plasma membrane. The α-subunits for the stimulatory (α_s) receptor and the inhibitory (α_i) receptor are non-identical but highly homologous. These hydrophilic subunits bind guanine nucleotides GDP and GTP, possess GTP-ase activity and have recognition sites for the enzyme or effector protein whose activity they regulate. The activity of the α-subunit is controlled by the hydrophobic $\beta\gamma$ complex, which is only separated under denaturing conditions. In the resting state GDP is bound to the G-protein maintaining its inactive form in association with the receptor. Following agonist binding to its specific receptor, GDP is exchanged for GTP and the G-protein dissociates into the $\beta\gamma$ complex with the active α-subunit modulating effector activity. The intrinsic activity of the α-subunit hydrolyses GTP to GDP, promoting dissociation from effector and reassociation with the $\beta\gamma$ subunits. Stable GTP analogues, GTPγs and GMP-PNP allow prolonged activation of G-proteins since they are non-hydrolysable, maintaining the α-subunit in its active form. Prostanoids act via receptors that are directly coupled to specific G-proteins, the biochemical outcome of receptor occupancy depending on the nature of the G-protein with which the occupied receptor is able to interact. Bacterial toxins covalently modify the G-proteins by addition of an ADP-ribose group from NAD to specific sites on the α-subunit, and consequently are useful tools in determining the involvement of G-proteins in signal transduction. Cholera toxin (CTX) blocks the GTPase activity of α_s thereby maintaining it in its active form. Pertussis toxin (PTX) blocks the agonist-induced inhibitory effect of α_i by stabilising the α_i-GDP/$\beta\gamma$ complex and uncoupling G_i from the receptor (Ui, 1984).

There is evidence of a role for G-proteins in activation of PLC from both permeabilised platelets and human platelet membranes (Nozawa *et al.*, 1989). Stable GTP analogues reduce $[Ca^{2+}]_i$ necessary for platelet activation (Haslam & Davidson, 1984) and cause an increase in DAG formation and concomitant hydrolysis of phosphoinositides (Lapetina, 1986; Brass *et al.*, 1986). There

appears to be differing opinions as to whether there is a similarity between this G-protein mediating receptor coupling to PLC (G_p) and the G_i inhibiting adenylate cyclase in platelets.
Brass *et al.* (1986) and Williams *et al.* (1990) both report on a PTX-sensitive G-protein to be involved in the regulation of thrombin-induced phosphoinositide hydrolysis. This similarity of G_i and G_p is supported by a study using an antibody to the α_i subunit of G_i isolated from bovine brain, which is shown to cross-react with the protein involved in the formation of IP_3 and DAG as a result of collagen-induced platelet activation (Walker & Bourguignon, 1990). In contrast, Lapetina (1986) suggests that not only does PTX not inhibit the hydrolysis of phosphoinositides in response to thrombin, it increased hydrolysis, thereby suggesting an inhibitory influence of G_i on this platelet response to thrombin.

A report that thrombin stimulates PLC and inhibits adenylate cyclase, whereas adrenaline only inhibits adenylate cyclase, tends to suggest the G proteins mediating these two pathways cannot be the same (Haslam *et al.*, 1990). In the same study PTX or NEM, which has been shown to inactivate inhibition of adenylate cyclase by adrenaline (Jakobs *et al.*, 1982), almost completely blocked the inhibition of adenylate cyclase induced by adrenaline, whereas the response to thrombin in stimulating PLC was reduced by 50 % as was the inhibitory influence on adenylate cyclase. Since they have identified a guanine-nucleotide independent PLC activity in the presence of high concentrations of Ca^{2+} which could be partially inhibited by PTX or NEM, they conclude that most of the thrombin-stimulated PLC activity does not in fact require functional G_i. In relation to the stimulation of the PLC pathway by PAF and the TP-receptor agonist, U44069, Houslay *et al.* (1986) support not only the lack of involvement of a PTX-sensitive G-protein but also that of G_s through studies with both PTX and CTX. It has been suggested that there is a heterogeneous population of G_p proteins (Cockcroft, 1987). The PTX-insensitive G_p is distinct from G_i, with immunochemical studies suggesting the PTX-sensitive form is not actually identical to G_i.

Brass *et al.* (1988) suggest that platelet TXA_2 receptors are coupled to a toxin-resistant form of G protein distinct from the one that is coupled to thrombin receptors, though both agonists stimulate phosphoinositide metabolism. Therefore more than one G protein appears to couple receptors to PLC, which may account for some discrepancies.

Adenylate cyclase in platelets is regulated by the G-proteins G_s and G_i (Stiles & Lefkowitz, 1982; Gilman, 1984; Houslay, 1984; Katada *et al.*, 1984). G_i inhibits adenylate cyclase directly via α_i, and/ or indirectly through the binding of $\beta\gamma_i$ to α_s so inactivating the latter. Small molecular weight GTP-binding proteins have also been isolated from human platelet membranes (Ohmori *et al.*, 1989; White *et al.*, 1990). They may have a role in the coupling to PLC of the murine monoclonal antibody AG-1, which causes platelet activation (Kroll *et al.*, 1990). This antibody is generated by immunising mice with washed platelets from patients with platelet-type von Willebrand disease, a rare bleeding disorder in which von Willebrand's factor, normally synthesised by vascular endothelial cells and present in the platelets, is missing.

There is a negative correlation between the ability of aggregating agents to inhibit adenylate cyclase and stimulate PI turnover, so that agents that are potent activators of PLC, such as thrombin, PAF and vasopressin, are poor inhibitors of adenylate cyclase, whereas potent inhibitors of adenylate cyclase, such as ADP and adrenalin, activate PLC weakly. Receptors then, cannot be coupled simultaneousy to G_i and the G-protein controlling phospholipase activity, and the receptors for different aggregating agents may bind preferentially to one or the other. However if, as some groups are reporting, there is a functional similarity between these two G-proteins then some other factor may determine which receptors couple to which effector systems.

The stimulatory and inhibitory second messenger systems influencing platelet aggregation have been shown to interact with each other. In human platelets for instance, a rise in cyclic AMP inhibited the formation of inositol phosphates and diacylglycerol

(Nishizuka, 1984; Jakobs *et al.*, 1986). Since an increase in cyclic AMP reverses the rise in cytoplasmic Ca^{2+} caused by aggregating agents (Zavoica & Feinstein, 1984; Paul *et al.*, 1990), there must be an effect on Ca^{2+} sequestration in addition to the inhibition of Ca^{2+} mobilisation as a result of the inhibition of IP_3 formation. Protein kinase A, activated by cyclic AMP, was shown to inhibit PLC in human platelets (Walker & Watson, 1992).

Studies have suggested cyclic AMP to inhibit guanine nucleotide-induced activation of phosphoinositide-specific phospholipase C in human platelets (Yada *et al.*, 1989). This inhibition is reported to be mediated by A-kinase phosphorylating a membrane protein rather than the GTP-binding protein or PLC itself, possibly resulting in the uncoupling of the GTP-binding protein from PLC.

PKC, activated by diacylglycerol, reciprocally interferes with the adenylate cyclase system. This may involve phosphorylation of the $G_{i\alpha}$ subunit, thereby removing the stimulatory influence on aggregation occurring through the inhibition of adenylate cyclase (Jakobs *et al.*, 1986). The phosphorylation by PKC does not however, affect the ability of hydrolysis-resistant GTP analogues to inhibit adenylate cyclase, suggesting the receptor-G_i interaction is altered, rather than the G_i-adenylate cyclase. The GTPase activity of G_i may be the target (Gutierrez-Venegas & Garcia-Sainz, 1991). In contrast, Williams *et al.* (1987) suggest a predominant effect of PKC on the stimulation of cyclic AMP formation in intact platelets. Since the former study involved the use of membranes prepared from TPA-treated platelets, the observed differences may reflect the nature of the preparation. A rise in $[Ca^{2+}]_i$ may also inhibit both basal and PGE_1-stimulated adenylate cyclase in human platelet membranes, and the Ca^{2+} influx induced by the ionophore A23187 markedly inhibits the PGE_1-stimulated rise in cyclic AMP in intact platelets (Rodan & Feinstein, 1976).

Studies in our laboratory indicated that several PGE analogues could induce irreversible platelet aggregation (in part) through their interaction with TP-receptors (Jones *et al.*, 1979,1982). The presence of a 16-phenoxy group is particularly effective in

enhancing TP-receptor agonist potency. However the 16-phenoxy analogue, sulprostone, produced only small, slowly developing aggregation waves, but did enhance aggregation to other agents (eg. ADP and PAF). The enhancement was not affected by TP-receptor antagonists. Ranking of the agonist potency of a range of PGE analogues suggested that the EP_3-receptor could be involved (see enclosed paper). However, there were problems associated with the very high sensitivity of the IP-receptor - adenylate cyclase system in this functional assay (Jones & Wilson, 1990). For example, PGE_2 gave variable results in PRP from different donors. In some PRP's, potentiation of aggregation was observed at low PGE_2 concentrations (10 - 100 nM) with the log concentration-response curve becoming bell-shaped as the concentration of PGE_2 was increased. In other PRP's, inhibition of aggregation was seen, although the log concentration-response curve was always shallow. Opposing inhibitory and stimulatory actions on adenylate cyclase could account for these effects.

In an attempt to overcome these difficulties I have proposed that cyclic AMP measurements may be a better method of assessing the potentiating effects of PGE analogues. Cyclic AMP levels in human washed platelets were raised using a potent and stable IP-receptor agonist, cicaprost (Skuballa *et al.*, 1986; Sturzebecher *et al.*, 1986), and the abilities of PGE analogues to inhibit the rise were measured (proposed scheme as in Figure 3.1.3). Cicaprost was deliberately chosen as the cyclase activator since it lacks the potent EP_1-receptor agonist action of other prostacyclin analogues such as carbacyclin and iloprost (Dong *et al.*, 1986). Cicaprost does have some EP_3-agonist activity as judged by its ability to inhibit the twitch response of the guinea-pig vas deferens to electrical field stimulation (Lawrence *et al.*, 1992). However, it is quite a weak agonist (e.m.r. relative to sulprostone > 5000) and at the concentration of 8 nM used in the platelet assay, it is unlikely to interact significantly with EP_3- or any other EP- receptors.

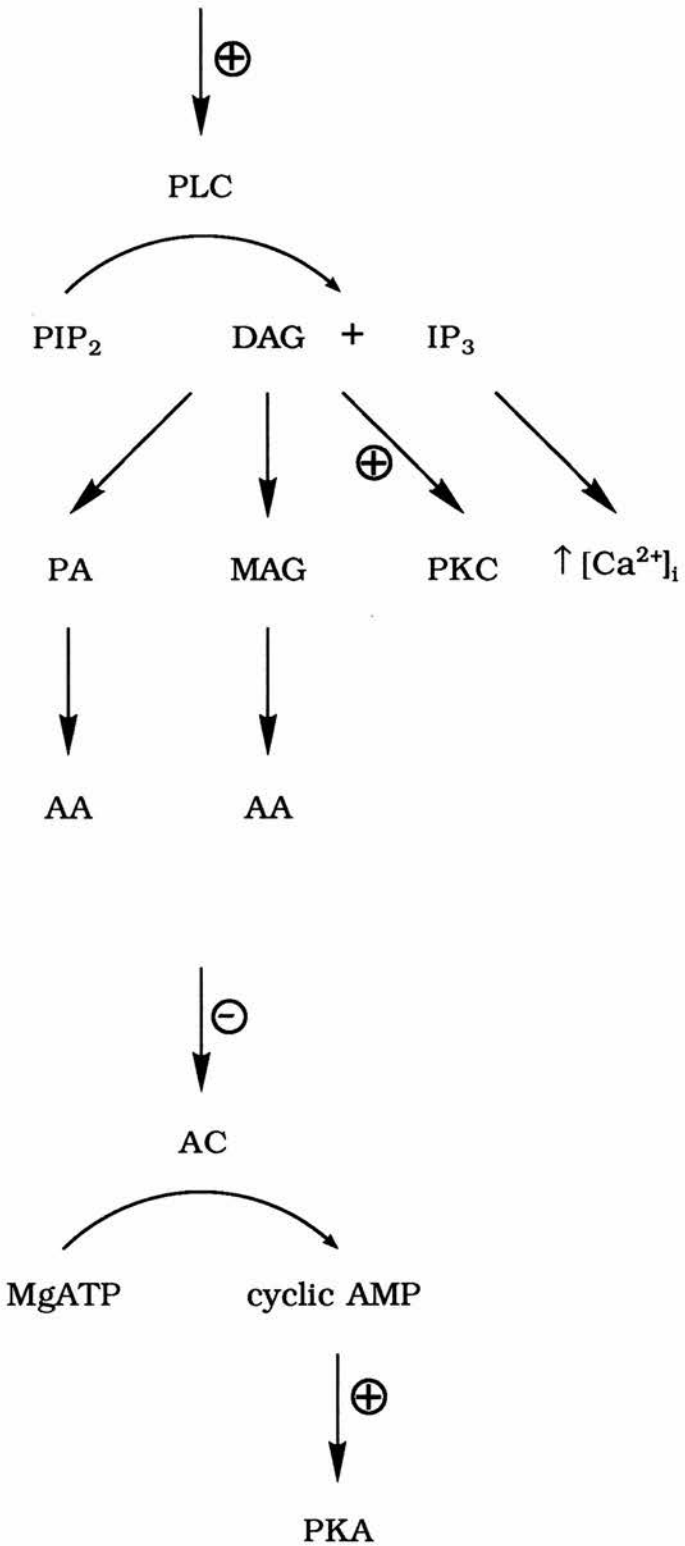


Figure 3.1.1 Stimulatory agonists of platelet aggregation

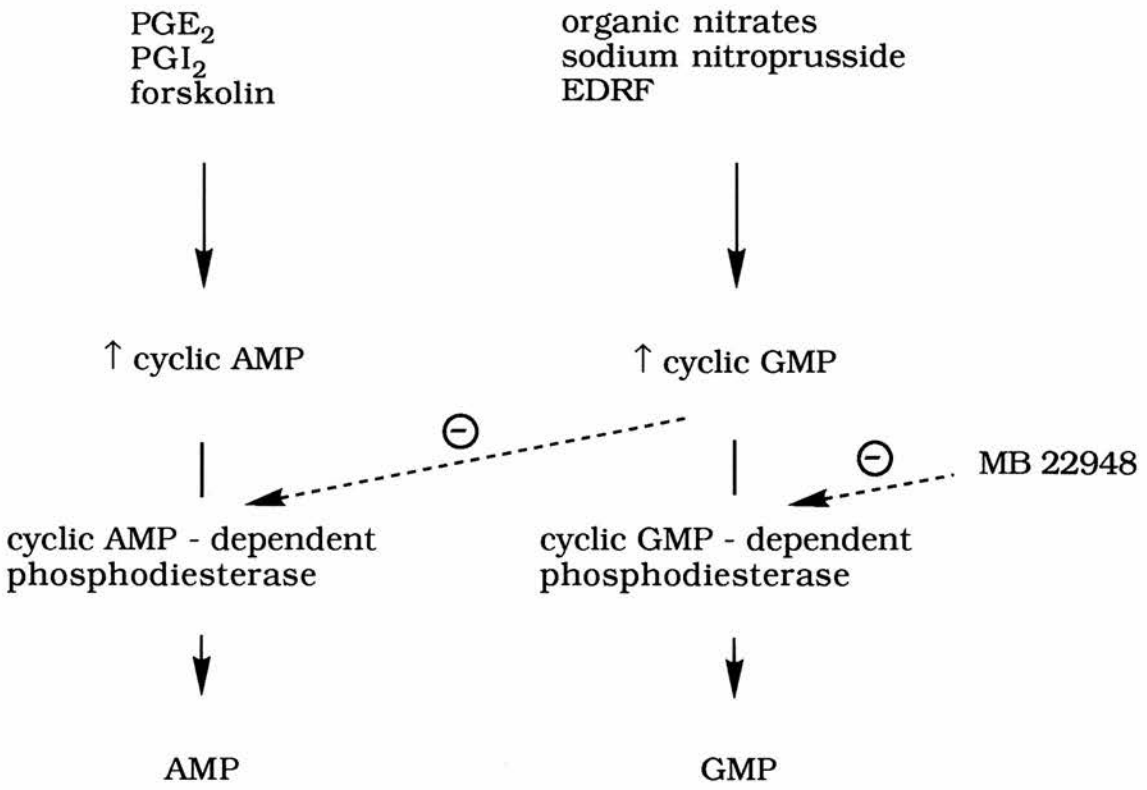


Figure 3.1.2 Inhibitory agonists of platelet aggregation

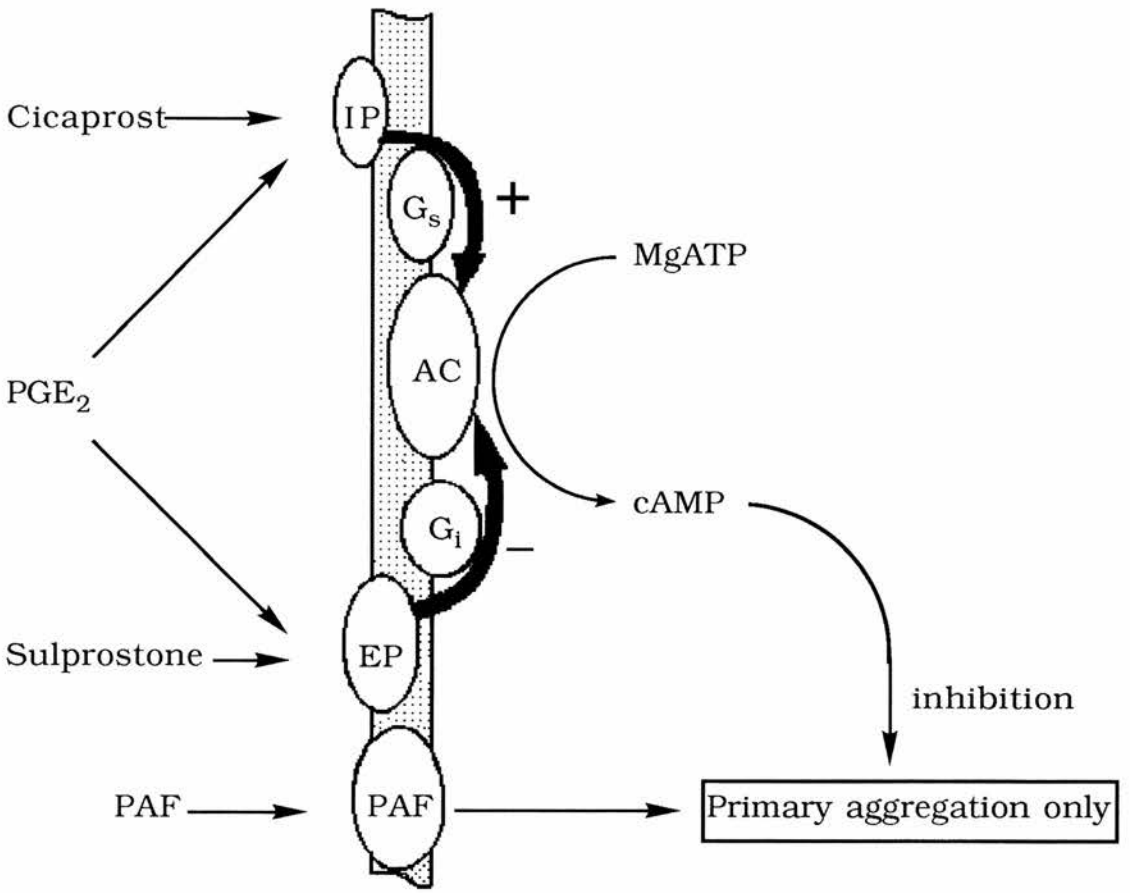


Figure 3.1.3 Interactions of prostaglandin receptors on the human platelet

3.2 MATERIALS and METHODS

SOLUTIONS		Source
Acid-Citrate Dextrose (ACD)		
6g	D-glucose	B.D.H. Chemicals
4g	disodium hydrogen citrate	"
- in 240 ml distilled water.		
Tris - HCl 500 mM pH 7 .4		
121.14 g	tris(hydroxymethyl)methylamine	"
~ 40 ml	concentrated HCl	"
- made up to 2 litre with distilled water.		
Tris (50mM) - EDTA (4mM)		
100 ml	tris - HCl (500mM pH 7.4)	
1.48 g	ethylenediaminetetraacetic acid (disodium salt)	"
- made up to 1 litre with distilled water.		

CHEMICALS	Source
AH 6809	Dr. R.A. Coleman, Glaxo, U.K.
GR 32191	"
GR 63799X	"
butaprost	Dr. P. Gardiner, Bayer, U.K.
cicaprost	Prof. H. Vorbruggen, Schering AG, Berlin
sulprostone	"
11-deoxy PGE_2-1-alcohol	Edinburgh (starting material *nat* PGA_2)
16,16-dimethyl PGE_2	Cayman Chemicals, U.S.A.
PGE_2	"
17-phenyl-ω-trinor PGE_2	"
indomethacin	Sigma (I 7378)
MB 28767 (racemic)	Dr. M. Caton, Rhone-Poulenc, U.K.
misoprostol	Dr. P. Collins, G.D. Searle, U.S.A.

REAGENTS

Prostanoids

Ethanolic stock solutions (10^{-2} - 3 x 10^{-2} M) were stored at -20 °C, and with the exception of GR 63799X, diluted with 0.9 % NaCl solution for use. Due to the insolubility of GR 63799X in 0.9 % NaCl, dilutions from stock were made in ethanol to ~ 2 x 10^{-4} M.

Cyclic AMP standards

Cyclic AMP dilutions were prepared in tris-EDTA assay buffer from a 20 mM stock, covering a range of 0 - 100 pmoles cyclic AMP / 50 µl for determination of the standard curve. The solutions were stored at -18 °C for a maximum of two weeks.

Binding protein

1.5 ml B.D.H. stock cyclic AMP - dependent protein kinase (product44199) was added to 20 ml tris-EDTA assay buffer. This was sufficient for 200 tubes and was prepared fresh for each assay.

[^{3}H] cyclic AMP

5 µl [8 - ^{3}H] Adenosine 3',5' - cyclic phosphate, ammonium salt from Amersham (TRK 304; 21.2 Ci/mmol, 1mCi/ml) was added to 10 ml tris-EDTA assay buffer. This supplied 200 tubes with 0.025 µCi each and the solution was stable for at least one month at -18 °C.

Charcoal adsorbent

0.52g B.D.H. charcoal (product 44199)
0.4g B.D.H. bovine serum albumin (product 44155)
- in 20 ml distilled water (per 200 tubes) -
Prepared fresh for each assay.

Procedure for extraction of cyclic AMP

The fresh blood donor pool comprised 4 female and 12 male healthy volunteers in the age range 21 - 46 years. No information was available for the time-expired platelets received from BTS.

1. 180 ml fresh blood collected by venepuncture from a human forearm was added to ~20% volume of acid - citrate dextrose solution and centrifuged at 250 g for 20 min.

2. 10 μM indomethacin was added to the platelet rich plasma (PRP) which was centrifuged at 450 g for a further 20 min to obtain a platelet pellet.

3. Time-expired (5-day old) human platelet concentrates (gratefully received from Blood Transfusion Service attached to the Royal Infirmary of Edinburgh) required only the second centrifugation step for platelet pellet preparation.

4. The pellet was suspended in 0.05 M tris buffer pH 7.4 containing 4 mM EDTA., at 37 °C in the presence of the TP-receptor antagonist GR 32191 (500 nM). The pellet obtained from 180 ml blood was resuspended in approximately 45 ml tris-EDTA buffer. Initially, quantitation of the platelet content of the suspension involved measurement of the absorbance at 600 nm (10 mm pathlength), giving a value of 1.4 ± 0.04 (n=16) for fresh platelets, and 1.4 ± 0.06 (n=4) for time-expired platelets. Subsequently, protein content was determined by the Bradford method (1976) using Pierce Coomassie Protein Assay Reagent (No. 23200), and gave 1.5 ± 0.1 mg/ml (n=10) for fresh platelets.

5. 0.5 ml aliquots of the platelet suspension were incubated with the PGE analogue for 2 min, followed by 8 nM cicaprost for 1 min, and the reaction quenched by the addition of 1 ml of ethanol.

6. The samples were centrifuged at 1000 g and 4 °C for 30 min, the ethanol supernatant decanted, evaporated to dryness at 55 °C under a stream of air and stored at -18 °C until assayed for cyclic AMP content.

3.2.1 Procedure for assay of cyclic AMP

Using the B.D.H. cyclic AMP kit and [^{3}H]-cyclic AMP from Amersham, the unknown samples were assayed based on the competitive binding of tritiated cyclic AMP and unlabelled cyclic AMP to a protein kinase isolated from bovine adrenal cortex. Separation of protein-bound cyclic AMP from the unbound nucleotide was achieved by adsorption of free nucleotide onto charcoal, followed by centrifugation. An aliquot of the supernatant was removed for liquid scintillation counting, giving an estimate of bound [^{3}H]-cyclic AMP. A standard curve allowed determination of unknowns. The procedure is summarised below.

1. Samples were resuspended in 250 µl tris-EDTA buffer and insoluble material sedimented by centrifugation at 3100 rpm (2270g) and 4 °C for 30 min. 50 µl aliquots of the supernatant were then assayed in duplicate.

2. The cyclic AMP assay was set up as outlined in Table 3.1. The tubes were whirlimixed and left on ice in the cold room for at least two hours.

3. The charcoal adsorbent was prepared at least 30 min before use, being continuously stirred with a magnetic stirrer and maintained at 4 °C during this time.

4. After the incubation period, 100 µl of charcoal adsorbent was added to 12 tubes at a time, whirlimixed and centrifuged at 3100 rpm (2270 g) and 4 °C for 2.5 min. (The charcoal should not remain in contact with the protein-bound cyclic AMP for more than 6 min, in order to minimise its dissociative effect on protein-bound cyclic AMP. Adding adsorbent to only 12 tubes at a time ensures that this time schedule is maintained).

5. A 200 µl sample of the supernatant was added to 5 ml Packard Emulsifier Safe scintillation fluid (No. 60143389), whirlimixed and counted on a Packard Liquid Scintillation Analyzer 1900CA.

6. A calibration curve (Figure 3.1) of % [^{3}H]-cAMP bound *vs* pmoles cyclic AMP was constructed from which unknown amounts of cyclic AMP in the extracted samples were determined.

Table 3.2.1 *Set-up for cyclic AMP assay*

Tubes	Description	Buffer / µl	cAMP / µl	sample / µl	[^{3}H] - cAMP / µl	binding protein / µl
1-3	charcoal blank	150	-	-	50	_
4-6	0 pmol cAMP	50	-	-	50	100
7-9	0.3 "	-	50	-	50	100
10-12	0.6 "	-	50	-	50	100
13-15	1.25 "	-	50	-	50	100
16-18	2.5 "	-	50	-	50	100
19-21	5 "	-	50	-	50	100
22-24	10 "	-	50	-	50	100
25-27	30 "	-	50	-	50	100
28-30	100 "	-	50	-	50	100
31, 32	unknown	-	-	50	50	100
33, 34	unknown	-	-	50	50	100
n_{-5}-n_{-3}	10 pmol cAMP	-	-	-	50	100
n_{-2}-n	1.25 "	-	-	-	50	100
T, T, T	total counts	250	-	-	50	-

Note:

- The charcoal adsorbent is not 100 % efficient in removing unbound cyclic AMP, and this is accounted for in tubes 1-3, charcoal blank. The average DPMs remaining in these samples of supernatant are subtracted from all the other counts.

- Two standards of cyclic AMP are included at the end of the assay in order to ensure the efficiency of binding or adsorbing has not altered throughout the course of the experiment.

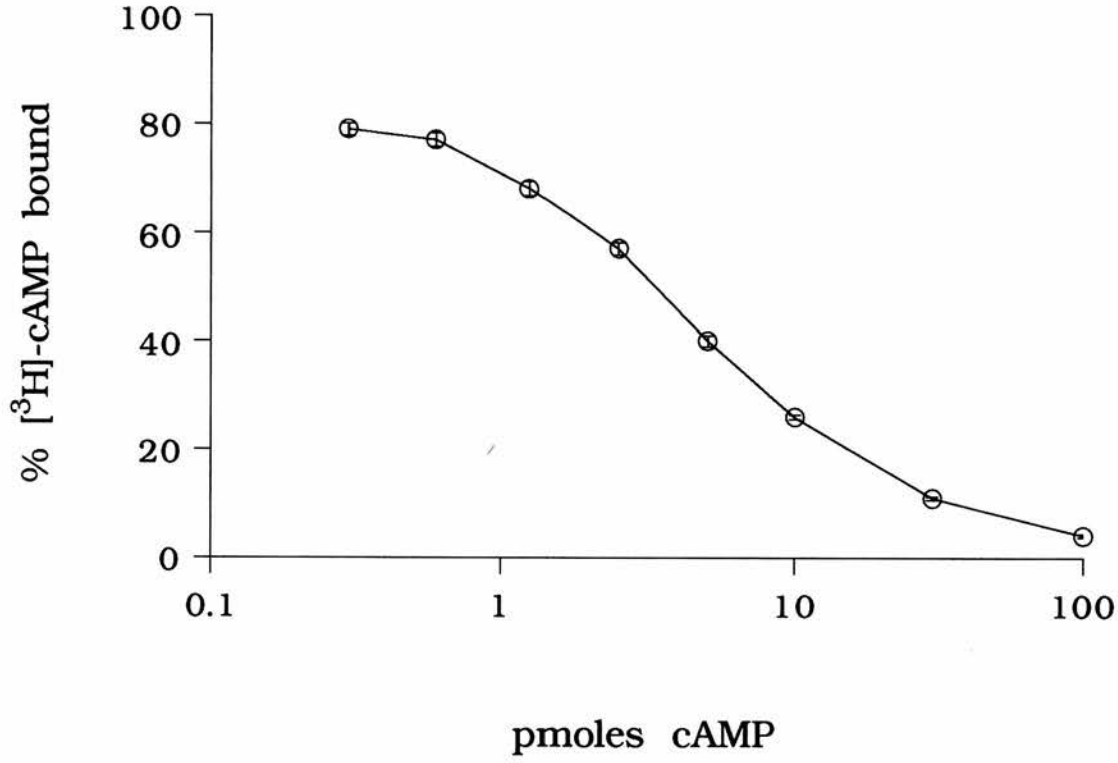


Figure 3.2.1. Calibration curve for determination of cAMP content in extracted samples. Each point is the average of 8 experiments. Vertical bars represent s. e. mean.

3.3 RESULTS

3.3.1 Efficiency of cyclic AMP extraction and assay

Cyclic AMP levels in human washed platelet suspensions were measured by competition with radiolabelled cyclic AMP for a cyclic AMP - dependent protein kinase. Recovery experiments were carried out to measure the efficiency of the extraction and assay protocols. Adding known amounts of cyclic AMP to the human washed platelet suspension followed by extraction and assay as previously detailed, gave an overall % recovery of 85 ± 5 (n=5). A known amount of [^{3}H]-cyclic AMP was also added to the human washed platelet suspension and an aliquot of the extracted sample counted in scintillation fluid. The recovery for this extraction process alone was 94 ± 3 % (n=5). The extraction and assay procedures are therefore efficient in the determination of cyclic AMP levels.

3.3.2 Fresh human washed platelets compared to time-expired

Due to the small size of our blood donor pool, some studies were also conducted on 5-day old stored platelets, gratefully received from the Blood Transfusion Service. With the exception of the time-course (Figure 3.2), the results represent the mean increase of cyclic AMP above basal levels, the vertical bars representing the standard error of the mean. Differences in cyclic AMP levels were assessed according to the Student's t -test, significance being accepted with values of probability less than 0.05. Basal levels were determined by incubation with saline and were
25 ± 7 pmoles cyclic AMP / ml platelets (n=6) and
7 ± 2 pmoles cyclic AMP / ml platelets (n=3) for fresh and time-expired platelets respectively.

3.3.3 8 nM cicaprost for 1 min represents control cyclic AMP

Cicaprost elevates cyclic AMP levels in the platelet through

interaction with the IP-receptor. The concentration-response curve to cicaprost (Figure 3.1) illustrated there was no significant difference between fresh and time-expired platelets at any of the concentrations tested over the range 3 nM to 800 nM, as represented by 54 ± 15 to 206 ± 46 pmoles / ml platelets (n=5-6) and 51 ± 22 to 187 ± 68 pmoles / ml platelets (n=3) respectively. To provide a sensitive system for the assessment of inhibitory effects on cyclic AMP production, an appreciable but submaximal level of cyclic AMP was required. 8 nM Cicaprost incubated with the human washed platelet suspension for 1 min produced 105 ± 22 pmoles / ml platelets (n=6) representing 52 ± 5 % of the maximum and a 5.7 ± 1.6 - fold increase over basal levels for fresh platelets, and 95 ± 30 pmoles / ml platelets (n=3) representing 44 ± 0.01 % of the maximum and a 15 ± 7 - fold increase over basal levels for time-expired platelets.

The time-course (Figure 3.2) demonstrated a 1 min incubation period to produce 86 ± 7 % (n=4) of the maximum cyclic AMP produced in a 5 min period, and the increase of cyclic AMP over basal (82 ± 27 pmoles / ml platelets) was not significantly different from that demonstrated above for the concentration-response in fresh platelets.

3.3.4 Inhibition of control levels of cyclic AMP

The inhibitory effects of PGE analogues were determined by a 2 min period of incubation over the range of concentrations 0.2 nM to 0.9 μM, followed by a 1 minute incubation with 8 nM cicaprost. PGE_2 and GR 63799X alone at 0.9 μM and 0.5 μM respectively, only increased cyclic AMP levels above basal by 6.2 ± 3.5 pmoles / ml platelets (n=5) and -0.5 ± 1.6 pmoles / ml platelets (n=4) respectively.

The inhibition curves obtained with sulprostone emphasise the similarity of response between the fresh and time-expired platelets. The IC_{50} value (concentration of PGE analogue inhibiting 50 % of the cicaprost-induced elevation of cyclic AMP) for

sulprostone in fresh platelets (Figure 3.3) was 3.0 ± 0.4 nM (n=4), compared to that in the time-expired platelet preparation (Figure 3.4) of 4.5 ± 0.7 nM (n=5). Sulprostone was used as the standard agonist and in subsequent experiments was included as a control. At 22 nM sulprostone produced an average of 16 ± 3 % (n=10) of the cicaprost-induced elevation of cyclic AMP. This was not significantly different from the value of 19 ± 3 % (n=10), indicated by the sulprostone dose-response curve (Figure 3.3).

In the fresh preparations (Figure 3.3), 17-phenyl-ω-trinor PGE_2 was approximately 30-fold less potent than sulprostone (IC_{50} = 100 ± 40 nM, n=7). 10μM AH 6809, whilst having no significant effect alone, produced a rightward shift in both curves, giving dose ratios of 7.3 and 3.9 for sulprostone and 17-phenyl-ω-trinor PGE_2 respectively. The presence of AH 6809 significantly reduced inhibition of the cicaprost-induced elevation of cyclic AMP by 2 nM to 65 nM sulprostone and 0.3 μM 17-phenyl-ω-trinor PGE_2.

Figure 3.4 illustrates the 4 analogues tested on the time-expired platelet preparations. 16,16-Dimethyl PGE_2, IC_{50} = 4.7 ± 1.7 nM (n=4), demonstrated a potency comparable to that for sulprostone. MB 28767 was less potent at 15 ± 6.6 nM (n=5), whilst misoprostol in its methyl ester form was approximately 15 times less potent than sulprostone with an IC_{50} of 76 ± 27 nM (n=5).

11-deoxy PGE_2-1-alcohol was inhibitory at the higher concentrations (IC_{50} = 720 ± 170 nM, n=4), (Figure 3.5), whilst butaprost showed no significant inhibition of the elevated cyclic AMP. PGE_2 and GR 63799X both inhibited the rise in cyclic AMP with IC_{50} values of 8.6 ± 2.0 nM (n=4) and 57 ± 8 nM (n=4) respectively (Figure 3.6).

α_2 - Adrenoceptor agonists induce G_i-mediated inhibition of adenylate cyclase in human platelets (Jakobs *et al.*, 1978). A single experiment demonstrated that pre-incubation with 1 μM adrenaline completely inhibited the cicaprost-induced elevation of cyclic AMP in fresh washed human platelets. The specific α_2 - adrenoceptor agonists oxymetazoline and UK 14304 both at 1 μM, inhibited cicaprost action by 90 % and 93 % respectively.

3.3.5 PGE analogues in other biological systems - a comparison

Table 3.4.1 compares the potencies of the PGE analogues tested as inhibitors of the cicaprost-induced elevation of cyclic AMP, with those as potentiators of PAF-induced aggregation of human platelet-rich plasma (PRP) (see enclosed paper) and as inhibitors of the twitch response to electrical field-stimulation of the guinea-pig vas deferens.

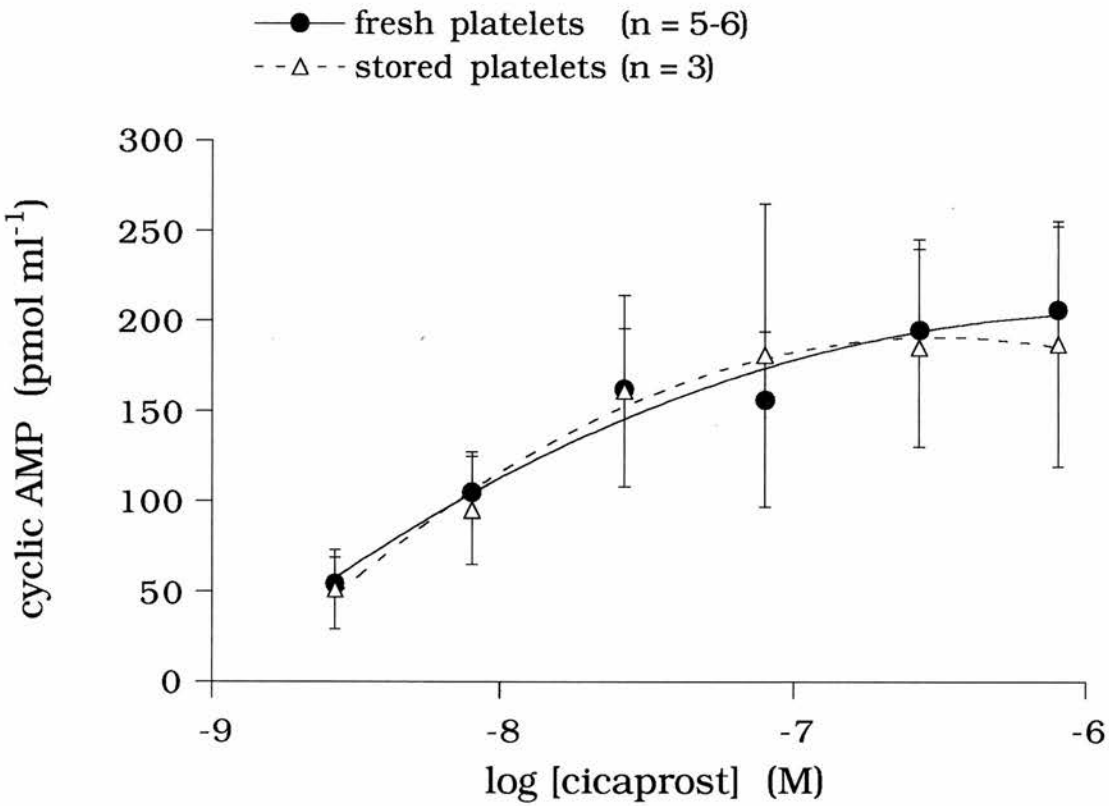


Figure 3.1 Accumulation of cyclic AMP in freshly prepared and time-expired suspensions of human washed platelets induced by increasing concentrations of cicaprost.

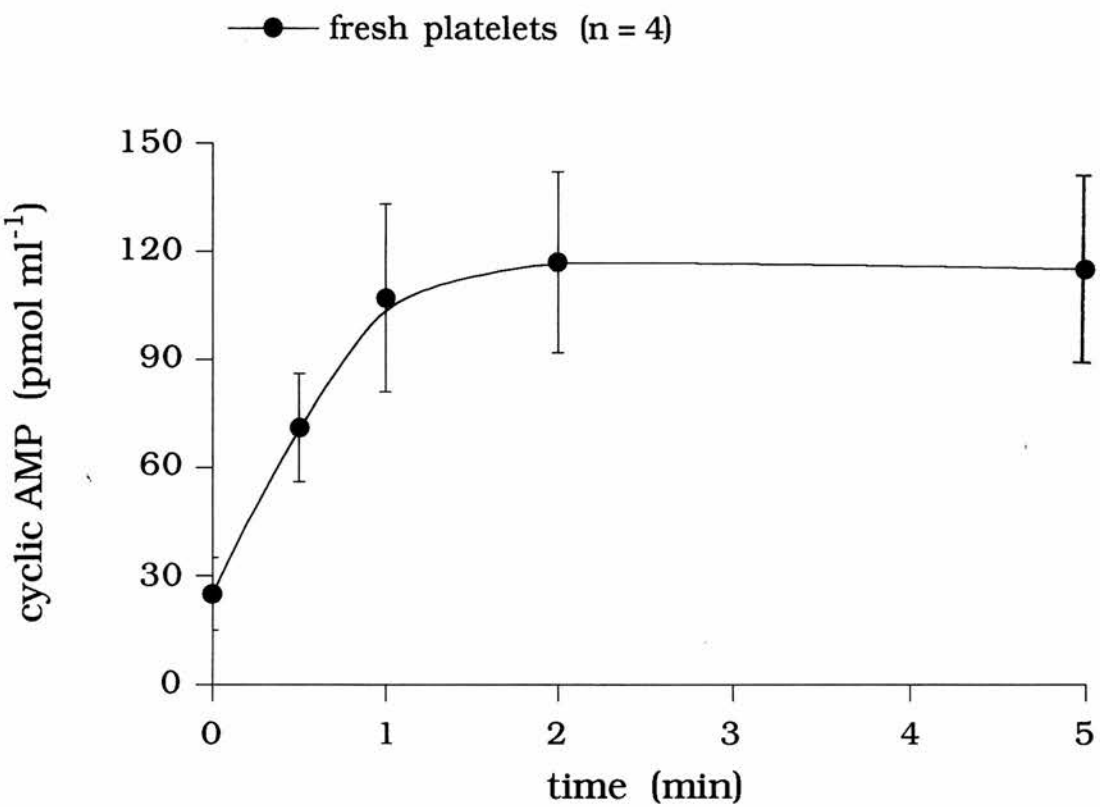
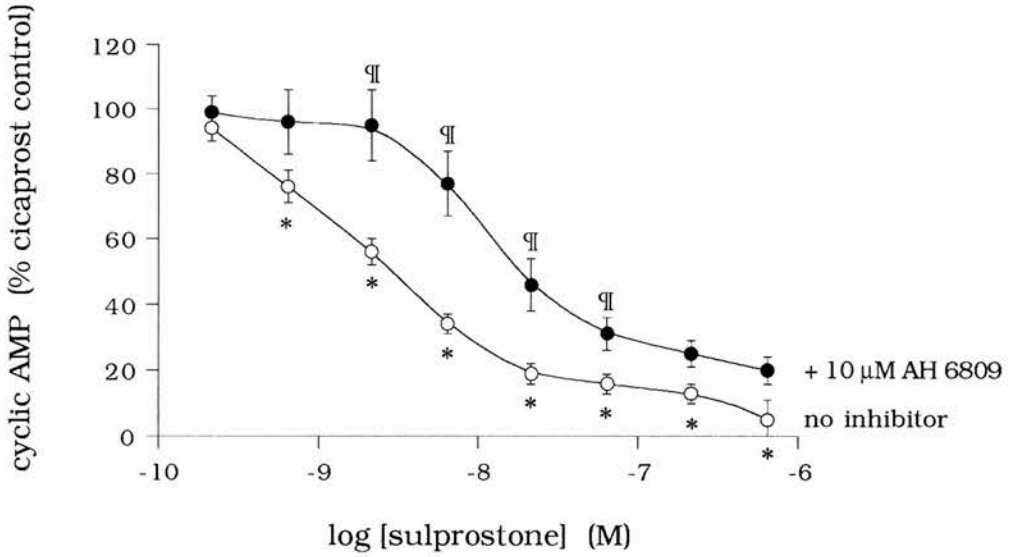


Figure 3.2 Time-course of cicaprost-induced elevation of cyclic AMP in freshly prepared suspensions of human washed platelets (n=4).

(a)



(b)

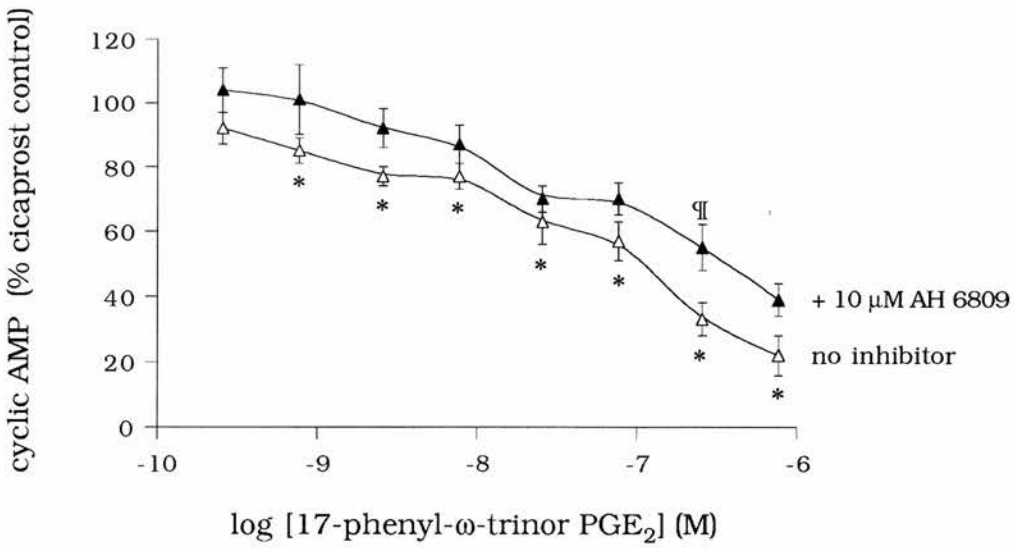


Figure 3.3 Inhibition of cicaprost-induced elevation of cyclic AMP in freshly prepared suspensions of human washed platelets as % cicaprost control, by :
(a) sulprostone (n=8-10), + 10 μM AH 6809 (n=4);
(b) 17-phenyl-ω-trinor PGE_2 (n=5-7), + 10 μM AH 6809 (n=5).

* denotes significant inhibition of cicaprost control ($p<0.01$);
¶ denotes significant difference from PGE analogue-induced inhibition ($p<0.05$).

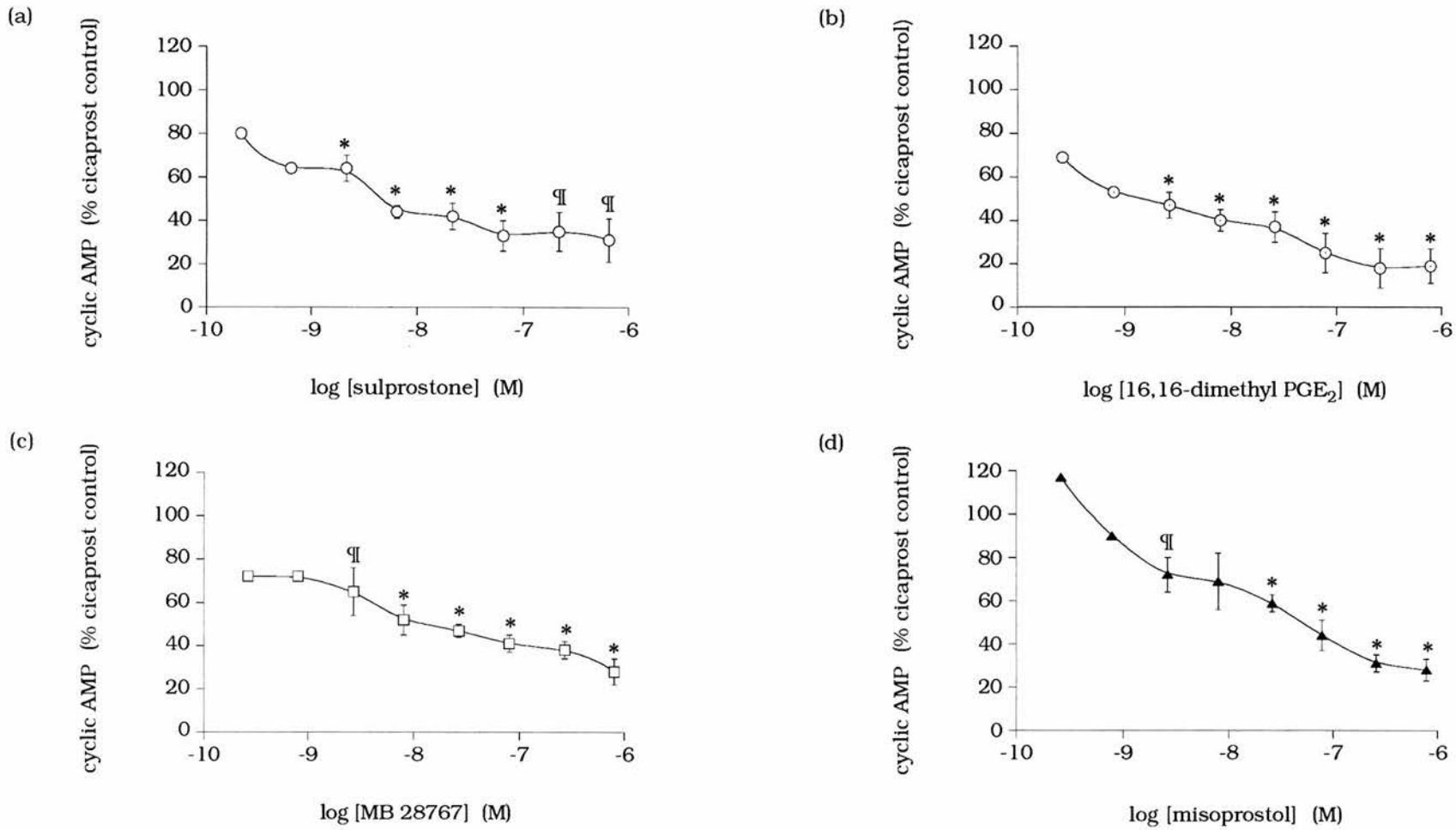
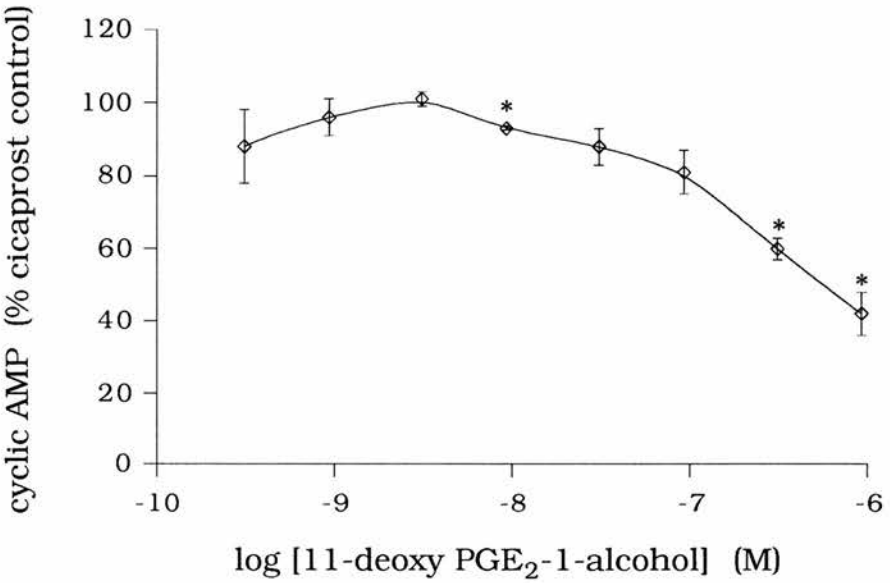


Figure 3.4 Inhibition of cicaprost-induced elevation of cyclic AMP in time-expired washed platelets as % cicaprost control, by : (a) sulprostone (n=2-5); (b) 16,16-dimethyl PGE_2 (n=2-5); (c) MB 28767 (n=2-5); (d) misoprostol (n=2-5).

Significant inhibition of cicaprost control is denoted by * ($p<0.01$) and ¶ ($p<0.05$).

(a)



(b)

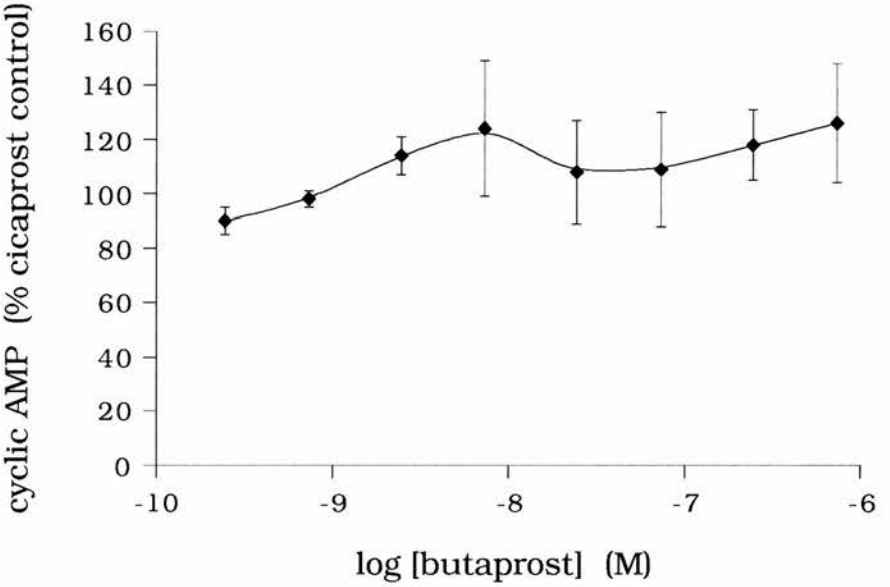


Figure 3.5 Inhibition of cicaprost-induced elevation of cyclic AMP in freshly prepared suspensions of human washed platelets as % cicaprost control, by :
(a) 11-deoxy PGE_2-1-alcohol (n=4), and
(b) butaprost (n=4).

* denotes significant inhibition of cicaprost control ($p<0.01$).

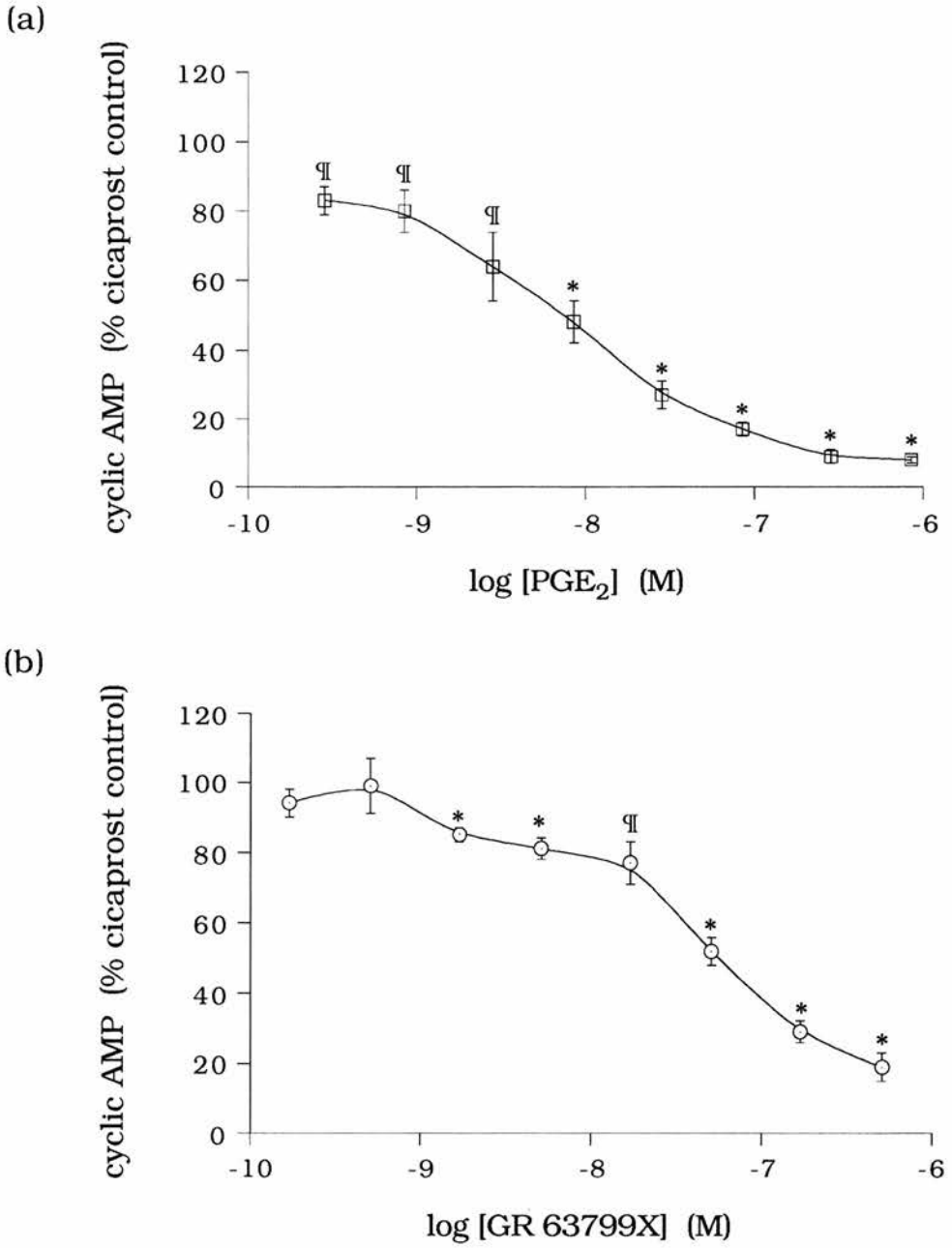


Figure 3.6 Inhibition of cicaprost-induced elevation of cyclic AMP in freshly prepared suspensions of human washed platelets as % cicaprost control, by :
(a) PGE_2 (n=4-5), and
(b) GR 63799X (n=4).

Significant inhibition of cicaprost control is denoted by * ($p<0.01$) and ¶ ($p<0.05$).

3.4 DISCUSSION

Cicaprost, a stable PGI_2 analogue (Skuballa *et al.*, 1986; Sturzebecher *et al.*, 1986) is known to increase cyclic AMP levels and exert its inhibitory influence on human platelets through interaction with the IP receptor (Dong *et al.*, 1986; Armstrong *et al.*, 1989). Incubation of human washed platelet suspensions with 8 nM cicaprost for 2 min at 37 °C produced a submaximal cyclic AMP response due to the specific IP-agonism at this concentration, and provided a sensitive system for the detection of PGE inhibitory effects. The pro-aggregatory activity of the PGE analogues which prompted a study into their inhibitory effects on cyclic AMP levels, were contemporaneous experiments carried out by a co-worker and may be found in the enclosed paper. The model which is being proposed for the pro-aggregatory effect of PGE_2 is shown (Figure 3.1.3).

Sulprostone, an EP_1- and EP_3- selective analogue (Coleman *et al.*, 1987a,b; Lawrence *et al.*, 1992), was shown to be the most potent of the PGE analogues tested in inhibiting the cicaprost-induced elevation of cyclic AMP in both fresh and time-expired platelet suspensions. The IC_{50} values are comparable at 3.0 ± 0.4 nM and 4.5 ± 0.7 nM respectively, suggesting EP-receptor coupling to adenylate cyclase has not altered after 5 days of storage at room temperature. The results of pro-aggregation similarly demonstrate sulprostone to be the most potent, at 10 nM producing a response representing 150 % (EC_{150}) of the control small reversible waves to PAF. Sulprostone has been shown to inhibit the increased cyclic AMP levels induced by the less specific IP-receptor agonist iloprost (Dong *et al.*, 1986), with an IC_{50} of approximately 100 nM (Thierauch & Prior, 1990). This low potency of sulprostone in comparison to the results already presented here, may be the result of the choice of agonist to increase the cyclic AMP level, the shorter pre-incubation time with the PGE analogue or the fact that PRP has been used.

17-Phenyl-ω-trinor PGE_2 is predominantly an EP_1-receptor agonist, having only moderate EP_3 agonist potency (Lawrence *et al.*, 1992) and even less activity at the EP_2-receptor (Dong *et al.*, 1986; Lawrence *et al.*, 1989; Lawrence *et al.*, 1992). It is therefore a useful tool in assessing the contribution of EP_1-activity to the sulprostone response. In comparing IC_{50} values, 17-phenyl-ω-trinor PGE_2 (IC_{50} = 100 ± 40 nM) is much less potent than sulprostone in inhibiting platelet cyclic AMP production. This suggests the EP_3 component of sulprostone activity to be the more important in the inhibition of cyclic AMP and is supported by studies carried out using the EP_1 antagonist (Coleman *et al.*, 1985a), AH 6809, at 10μM. Experiments with sulprostone and 17-phenyl-ω-trinor PGE_2 paired in the presence and absence of AH 6809, demonstrate a rightward shift in both dose-response curves with dose ratios for sulprostone and 17-phenyl-ω-trinor PGE_2 of 7.3 and 3.9 respectively. The Schild equation describes the relationship between the dose ratio and the concentration of antagonist by:

$$\text{dose ratio (DR)} - 1 = [\text{antagonist}] / K_d \text{ antagonist.}$$

If DR = 2, [antagonist] = K_d antagonist, therefore the pA_2 = -log (K_d antagonist). Values of pA_2 for sulprostone and 17-phenyl-ω-trinor PGE_2 are calculated as 5.8 and 5.5 respectively. This is in contrast to the reports of AH 6809 as an antagonist on EP_1 preparations where it is 10 - 100 times more potent, as demonstrated by a pA_2 of 6.8 - 7.5 (Coleman *et al.*, 1987c; Eglen & Whiting 1988; Lawrence *et al.*, 1992). The relative potencies of sulprostone and 17-phenyl-ω-trinor PGE_2 and the weak antagonistic effect of AH 6809 on each, suggests the EP_3 component of sulprostone activity to be the important factor in the inhibition of the cicaprost-induced increase in cyclic AMP levels. Indeed, 17-phenyl-ω-trinor PGE_2 itself has a weak EP_3 effect as assessed by inhibition of the twitch response of the guinea-pig vas deferens to electrical field stimulation (Lawrence *et al.*, 1992). In this EP_3-preparation the reported e.m.r. of 17-phenyl-ω-trinor PGE_2 relative to sulprostone is 45, and correlates well with the

e.m.r. of 33 for the inhibition of cicaprost-induced elevation of cyclic AMP reported here. Therefore the EP_1 component of 17-phenyl-ω-trinor PGE_2 and sulprostone appears to contribute minimally if at all to their inhibitory effect. These studies are in agreement with the functional assay where 17-phenyl-ω-trinor PGE_2 was reported to be 29 times less potent than sulprostone (EC_{150} = 10 nM) in potentiating aggregation.

GR 63799X is a novel potent selective EP_3-receptor agonist which has been tested for its inhibitory effect in this system. On EP_3-receptor containing preparations such as the guinea-pig vas deferens (Bunce *et al.*, 1990; see enclosed paper) and rat isolated gastric mucosa (Bunce *et al.*, 1990), it is 4 - 50 times more potent than PGE_2. However, in the inhibition of cyclic AMP levels, GR 63799X is 8 times less potent than PGE_2. A comparison of the potencies in potentiation of PAF aggregation is not possible due to the inhibitory effect observed for PGE_2 (see enclosed paper). It was however found to be 26 times less potent than sulprostone in potentiating PAF-induced aggregation.

Three other PGE analogues with EP_3 activity have been tested on this system using time-expired washed platelets. The good correlation between this and the fresh preparation as regards the activity of sulprostone, support a comparison of the PGE analogues irrespective of whether fresh or time-expired platelets. 16,16-Dimethyl PGE_2, having activity at all three classified EP subtypes (Dong *et al.*, 1986), showed a potency comparable to that of sulprostone as represented by IC_{50} = 4.7 ± 1.7 nM (e.m.r. = 1.6). This compares well with the e.m.r. for 16,16-dimethyl PGE_2 relative to sulprostone of 1.3 and 0.93 in the functional assay and the guinea-pig vas deferens respectively. The assessment of the EP-activity of MB 28767 is complicated by its agonist action at TP-receptors (Banerjee *et al.*, 1985). However, this TX activity may be blocked by routine treatment of the platelet suspensions with 500 nM GR 32191 (Lumley *et al.*, 1989). Under these circumstances MB 28767 demonstrated an IC_{50} of 15 ± 6.6 nM in inhibiting elevation of cyclic AMP levels. The IC_{50} for inhibition in the vas deferens is similarly 5-fold greater than that for

sulprostone, whilst the functional assay suggests a slightly less potent effect of MB 28767 (e.m.r. = 6.8). This may be a result of plasma protein binding of MB 28767. The aggregation experiments with washed platelets are difficult since the platelets lose potentiating activity, prefering their native environment. Consequently PRP is the preferred platelet preparation for the aggregation studies. In contrast to the three EP_3-selective analogues so far reported, misoprostol in its methyl ester form has a relatively high IC_{50} of 76 ± 27 nM. This agrees with the 13-fold less potent activity of misoprostol relative to sulprostone in potentiating aggregation, and may reflect the free acid form to be the more biologically active. Studies on the guinea-pig vas deferens do however indicate a greater potency of misoprostol (3.6-fold less) compared to sulprostone, than either of these studies on the platelet.

The EP_2-selective agonist butaprost (Gardiner, 1986) did not display any inhibition of cicaprost-elevated cyclic AMP levels approaching μM concentrations, whilst 11-deoxy PGE_2 1-alcohol was inhibitory at the higher concentrations with an IC_{50} of 720 ± 170 nM. This may reflect a degree of agonism at EP_3 receptors by 11-deoxy PGE_2 1-alcohol, which is completely absent in the case of butaprost (Lawrence *et al.*, 1992). The EP_2-receptor is classically coupled to adenylate cyclase through G_s, interaction with which results in an increase in intracellular cyclic AMP levels (Creese & Denborough, 1981). In the pro-aggregation studies, difficulties arose when using PGE_2, butaprost and 11-deoxy PGE_2 1-alcohol through inhibition of the aggregation response. The lack of effect of the EP_2-selective agonist butaprost, supports the opinion that PGE_2 and 11-deoxy PGE_2 1-alcohol display IP agonism, in the absence of an EP_2-receptor on the platelet. We reasoned that the effect of any weak IP agonism which complicates the functional assay, would be lessened by the increased cyclic AMP levels induced by cicaprost in this system. Inhibition of cyclic AMP levels therefore allows for better assessment of the potency of PGE analogues such as

11-deoxy PGE_2 1-alcohol and PGE_2, where inhibition is often seen in measuring potentiation of aggregation.

In spite of the difficulties incurred in the aggregation studies, PGE_2 is shown to be approximately 3 times less active than sulprostone in inhibiting the elevation of cyclic AMP levels. The greater potency of sulprostone relative to PGE_2 on EP_3 preparations has been reported elsewhere. For example on the guinea-pig vas deferens sulprostone is ~7 times more potent than PGE_2 in inhibiting the twitch response to electrical field stimulation (Lawrence *et al.*, 1992).

Table 3.4.1 summarises these results as equipotent molar ratios of the PGE analogues relative to sulprostone. These have been compared with results obtained from potentiation of PAF-induced aggregation in human PRP and inhibition of the twitch response in the guinea-pig vas deferens to electrical field stimulation (EP_3-receptor preparation). Comparison of the ranking of agonist potencies on these three systems suggests the PGE_2-induced potentiation of aggregation in platelets to be mediated by an EP_3-like receptor negatively coupled to adenylate cyclase.

An early report suggests coupling of an EP-receptor(s) to inhibition of adenylate cyclase, independently of extracellular Ca^{2+}, in the PGE_2-mediated reduction of catecholamine release from the adrenal medulla (Gutman *et al.*, 1979). The dual effect on cyclic AMP levels observed for platelets was also demonstrated on cultured cortical collecting tubule cells, where PGE_2 at low concentrations (> 10^{-10} M) was shown to reduce the cyclic AMP - elevating effect of arginine vasopressin, whilst at higher concentrations (> 10^{-9} M) PGE_2 independently stimulated cyclic AMP formation. Subsequently a solubilised complex of PGE receptor and PTX-sensitive guanine nucleotide regulatory protein, N_i, was isolated (Smith *et al.*, 1987). PGE_2 has more recently been reported to reduce elevated platelet cyclic AMP levels induced by PGI_2, though the absence of data in this abstract precludes comparison of a more quantitative nature (Faili *et al.*, 1992). It was suggested that this effect of PGE_2 is the result of competition with PGI_2 for the IP-receptor, which is unlikely in view of the evidence

presented in the introduction (section 3.1.4). supporting distinct receptors for PGI_2 and PGE_2.

The PGE-inhibitory receptors are apparently linked to adenylate cyclase through G_i (Ashby, 1986,1988). Pertussis toxin is not effective in intact platelets, as they lack the receptor to which a subunit of PTX binds in order to allow the catalytic unit to enter. However, phorbol ester results in PKC-mediated phosphorylation of G_i (Jakobs *et al.*, 1985; Katada *et al.*, 1985; Watanabe *et al.*, 1985) impairing its function and allowing the importance of G_i to be assessed. It would obviously be of great interest to assess the effect of such alterations to G_i in this system. In light of the interaction between the PLC and adenylate cyclase pathways as outlined in the introduction, there is the possibility that the reduction in cyclic AMP levels is indirectly the result of a stimulation of the PLC pathway. Indeed MacIntyre & Gordon (1975) suggest the pro-aggregatory effect of PGE_2 is calcium-dependent, based on the reduced potentiation of collagen-induced aggregation in citrated PRP as compared to heparinised PRP, both in rat and man. Thierauch & Prior (1990) do however find no influence of PMA-activated PKC on the iloprost-induced increase in cyclic AMP levels in PRP or gel-filtered platelets, nor any change in the $[Ca^{2+}]_i$ in gel-filtered platelets. It has recently been suggested that the inhibitory effect of PGE_2 on cardiac sarcolemmal adenylate cyclase is the result of coupling via a pertussis toxin sensitive G_i protein (Lerner *et al.*, 1992).

Bearing in mind the controversy over the similarity between G_i and G_p, the results of perturbations to G_i may not actually give any greater insight into the pathway of inhibition of adenylate cyclase. What would be of use, is to conduct the cyclic AMP studies on platelet membranes. The adenylate cyclase is membrane bound, therefore if this is directly coupled to the EP receptor via G_i, a similar effect to what is observed in intact platelets should be obtained. However, the water-soluble products of PIP_2 hydrolysis, formed as a consequence of the membrane-bound PLC activity, should be ineffective in the absence of the intracellular calcium

pools in the membrane preparation, thereby abolishing any inhibitory influence on adenylate cyclase which may occur indirectly via the PLC pathway. Perhaps a more in depth study of the mechanisms possible for the inhibition of elevated cyclic AMP levels may reconcile the finding discussed below, that inhibition of basal cyclic AMP levels do not appear to potentiate platelet aggregation in response to submaximal doses of the aggregating agents.

Inhibition of adenylate cyclase in the stimulation of platelets has been reported for other agonists (reviewed in Hourani & Cusack, 1991). ADP for instance has a role in the secondary aggregation induced by other aggregating agents which are responsible for its release from the granules. It inhibits adenylate cyclase though it is not clear if this is mediated by the same receptor which causes shape change, aggregation and the release of granule contents *in vitro*. Catecholamines such as adrenaline and noradrenaline, through an interaction with the α-receptor may also inhibit adenylate cyclase (Jakobs *et al.*, 1978). However. the concentration of adrenaline required for platelet activation *in vitro* (> 0.1 μM) is much higher than the levels found circulating in the blood (0.1 nM) (Culliver & Ardlie, 1981). The role of adrenaline may therefore be similar to that of PGE_2, such that while unable itself to cause platelet aggregation, can potentiate the platelet response to other agonists. Through the interaction with a single α_2-receptor, adrenaline may induce aggregation and inhibit adenylate cyclase previously stimulated by PGI_2 or PGE_1, both in intact platelets and platelet membranes. The mechanism of action of adrenaline is GTP-dependent, and the sensitivity to PTX and proteolytic treatment of the platelet membranes with chymotrypsin, which degrades G_i, suggests the involvement of G_i.

There is controversy as to whether a reduction in basal intracellular cyclic AMP concentrations as a consequence of adenylate cyclase inhibition can lead to platelet activation. Direct inhibition of the catalytic subunit of adenylate cyclase by

2',5'-dideoxyadenosine in intact platelets does not cause platelet aggregation, or even potentiation of platelet aggregation induced by other stimuli such as ADP, vasopressin, collagen or arachidonic acid. A decrease in the PGE_1-induced inhibition of ADP-induced platelet aggregation and the associated increases in cyclic AMP was however shown, demonstrating the ability to inhibit adenylate cyclase. The inhibition of adenylate cyclase is therefore thought to be responsible for potentiating aggregation in situations where the cyclic AMP levels have been raised. Perhaps stimulation of the catalytic subunit by G_s is required in order for G_i to interact, since it is only loosely associated (Levitzki, 1987). An inhibitory effect of PGE_2 on isolated bovine myometrial membrane has also been reported (Lerner *et al.*, 1990). Similarly, the inhibition of adenylate cyclase at 10^{-11} M was not expressed in membranes prepared in the absence of PGE_2. Alternatively, there may be pools of cyclic AMP within the platelet, the reduction of whose content exerting an effect whilst not reflected as a significant reduction of total basal cyclic AMP (Salzman, 1974).

Additional signalling mechanisms have been proposed for receptors negatively coupled to adenylate cyclase, with experimental evidence suggesting the reduction in cyclic AMP levels may not be the only mechanism (Limbird, 1988). For instance, in spite of desensitisation of aspirin-treated PRP to subsequent α_2-adrenergic receptor-induced aggregation, adrenaline-induced inhibition of PGE_1-stimulated cyclic AMP levels still occurred and adrenaline was able to inhibit basal adenylate cyclase activity in membranes prepared from desensitised platelets (Motulsky *et al.*, 1986). Radioligand binding studies also showed no change to the binding properties of the α_2-adrenergic receptor. Possibilities for the alternative signalling mechanisms which may be involved in situations where receptors are negatively coupled to adenylate cyclase, include Na^+ / H^+ exchange, and K^+ and Ca^{2+} channels. In a study conducted on rat hepatocytes, the synthesis of a cyclic AMP antagonist of low molecular weight (~ 1500) formed from PGE_2 has been proposed (Wasner, 1981). This intracellular, hormone messenger-like

regulator is formed in hepatocytes stimulated by insulin or adrenaline, and is reported to inhibit cyclic AMP - dependent protein kinases and activate phosphatases. It has since been renamed prostaglandylinositol cyclic phosphate (cyclic PIP) (Wasner *et al.*, 1991). There are therefore many additional possibilities to a direct inhibition of adenylate cyclase, for the mechanisms of stimulation and potentiation in human platelets.

Whilst there have been reports of inconsistencies between the inhibition of adenylate cyclase and the aggregation response tending to suggest additional mechanisms, the good correlation of the e.m.r. of a series of PGE analogues in inhibiting the increased cyclic AMP levels and potentiating the small reversible waves to PAF suggests an important involvement of this mechanism in the potentiation of aggregation in the human platelet to PGE_2. In addition, a further comparison of the activity of the analogues used with their effect in inhibiting the twitch response of the guinea-pig vas deferens to electrical field stimulation, suggests the receptor may be of the EP_3-subtype.

Table 3.4.1 Comparison of potencies of PGE analogues

Prostanoid	Equi-effective molar ratios (EMR) Human platelets ↓ cAMP washed	Human platelets ↑ aggregation PRP	Guinea-pig § vas deferens
Sulprostone	1.0 (IC_{50} = 3.0/4.5* nM)	1.0 (EC_{150} = 10 nM)	1.0 (IC_{50} = 0.2 nM)
16,16-Dimethyl PGE_2	1.6*	1.3	0.93
PGE_2	2.9	↓	7.1
MB 28767	5.0*	6.8	5.0
GR 63799X	19	26	1.7
Misoprostol	25*	13	3.6
17-Phenyl-ω-trinor PGE_2	33	29	45
11-Deoxy PGE_2-1-alcohol	240	↓	79
Butaprost	>320	↓	>7000

* stored platelets

↓ inhibition of aggregation often seen

§ data from Lawrence *et al.* (1992), with the exception of GR 63799X

Chapter 4

Characterisation of the PGE receptor on human macrophage-like cells through binding studies conducted on a purified plasma membrane preparation

4.1 INTRODUCTION

4.1.1 Prostaglandins are *modulators* of inflammation

Prostaglandins are widely recognised as mediators of inflammation and inhibitors of their synthesis are therapeutically useful anti-inflammatory agents (Ferreira *et al.*, 1971; Smith & Willis 1971; Vane 1971). However, depending on the experimental situation studied, PGE_2 displays either pro- or anti- inflammatory effects (Bonta & Parnham, 1978, 1980; reviewed in Goodwin, 1991). Indeed stable prostaglandins such as misoprostol and PGE_1 acted synergistically with NSAIDs in the inhibition of neutrophil activation (Weissmann, 1992). PGE_2 may therefore be more accurately described as a modulator of inflammation.

Of the cells involved in inflammation, the vascular endothelial cells, mast cells and tissue macrophages are present in the tissues, the platelets and leucocytes gaining access from the blood. The white blood cells or leucocytes can be subdivided into polymorphonuclear cells, or granulocytes, and mononuclear cells. The former consist of neutrophils, eosinophils and basophils, the mononuclear cells comprising monocytes and lymphocytes. Monocytes circulate in the blood for up to 1-4 days and differentiate for example into macrophages (Kupffer cells) in liver, into alveolar macrophages in lung and into peritoneal macrophages in peritoneum, where they may remain for periods of up to several months.

The inflammatory reaction consists of both immunologically specific reactions and innate reactions having no immunological basis. The latter involves an increase in vasodilatation and vascular permeability, release of mediators from vascular endothelial cells, mast cells and platelets, and phagocytosis of dead cells, tissue debris and foreign material by leucocytes. The polymorphonuclear cells are the first of the blood leucocytes to enter the area of the inflammatory reaction, where they engulf, kill and digest the invading microorganism. Macrophages secrete a range of

substances including enzymes, plasma proteins and substances regulating the function or growth of other cells (Takemura & Werb, 1984). A variety of enzymes participate in a wide range of functions, such as accelerating inflammation, clearing up debris in inflammation sites, killing tumours and metabolising lipoproteins and may be regulated within the environment surrounding the macrophage by the release of enzyme inhibitors. Plasma proteins also participate in inflammation and are involved in tissue repair, immunoregulation and molecular transport. Low molecular weight substances such as reactive oxygen metabolites and bioactive derivatives of arachidonic acids are not strictly secretory products as they are released extracellularly after triggering by phagocytosis or chemical stimulation. The oxygen metabolites participate primarily in the extracellular killing of microbes and tumours.

In some tissues however, macrophages have a role in presenting antigenic (foreign) material to the lymphocyte, thereby initiating the specific immunological response. Those lymphocytes which recognise the antigen, multiply and differentiate into either of the two subsets of lymphocytes, B-cells, responsible for antibody production, or T-cells, involved in cell-mediated reactions. This facilitates a response more selective for the invading organisms.

The pro-inflammatory action of the prostaglandins is discussed in Chapter 2 of this thesis. Their anti-inflammatory action is less well recognised. In fact PGs of the E series can suppress many parameters of the immune response, including proliferative responses, antibody and lymphokine production and cytolysis (reviewed in Goodwin & Webb, 1980). Such suppression of the immune system is of great benefit in skin-grafting, where studies suggest an increase in PGE_2 may play a regulatory role in preventing rejection through a reduced response (Dy & Astoin, 1980). A suppressed immune system is also important in preventing the triggering of an inappropriate immune response in the lung, for instance, where there is daily exposure to a considerable variety of antigens (Monick *et al.* 1987).

When the host defence system functions efficiently, antigens are disposed of without compromising host function and well-being. However, in chronic inflammatory diseases such as rheumatoid arthritis, granulocytes and macrophages are involved in mediating the acute symptoms of swelling, erythema and pain, and the chronic changes which include local connective tissue proliferation, fibrosis and the irreversible destruction of articular cartilage. In addition, suppressor cells have been suggested as aetiologic or contributory in the pathogenesis of several diseases such as cancer (Goodwin *et al.*, 1977a) and Hodgkins' disease (Elkashab & Lala, 1991). Research into the mechanisms of signalling in the immune system is therefore of great interest and potential benefit to many.

4.1.2 IL-1 facilitates the immune response

Interleukins (ILs) are involved in the signalling between cells of the immune system (see Figure 4.1.1(a)). The macrophage product IL-1, or lymphocyte activating factor (LAF), causes proliferation of the lymphocytes. The mechanism of this lymphoproliferative effect appears to be mediated by stimulation of the release of IL-2 by T-cells in both human (Smith *et al.*, 1980; Maizel *et al.*, 1981) and murine models (Smith *et al.*, 1980). More specifically, IL-1, is produced by macrophages and other antigen-producing cells, acting on T-cells to induce IL-2 receptors. IL-2, or T Cell Growth Factor produced by these activated T-cells is necessary for their long term proliferation (Mizel, 1987), thereby amplifying their helper and cytolytic functions. By controlling IL-2 production then, an appropriate immune response should be triggered.

Studies have shown an absolute requirement for ~ 1 % monocytes for lymphocytes to produce IL-2 (Neefe *et al.*, 1981), suggesting depletion of monocytes from a mononuclear cell population would inhibit IL-2 production by the lymphocytes. However, in man a simple depletion by adherence of monocytes actually enhances

IL-2 production. In fact excess monocytes are capable of inhibiting IL-2 production, an effect mediated by soluble products and blocked by treatment with indomethacin (Chouaib & Fradelizi, 1982). Monocytes must therefore have a regulatory role in lymphocyte IL-2 production, exerting both stimulatory and inhibitory influences. The stimulatory component is only demonstrated when following almost complete depletion, achieved by a three step process of plastic adherence, filtration through a nylon wool column and cell lysis, IL-2 production is inhibited (Chouaib & Fradelizi, 1982).

4.1.3 PGE_2 inhibits mononuclear cell IL-1 production.

The IL-1 produced by the macrophage, besides inducing IL-2 production by the lymphocyte, also stimulates the release of PGE_2 from other macrophages (Figure 4.1.1 (b)). Stimulation of cultured mouse peritoneal macrophages by IL-1 resulted in a release of arachidonic acid from both phosphatidylcholine and phosphatidylinositol suggesting the involvement of both PLA_2 and PLC respectively (Watson & Wijelath, 1990). The PGE_2 formed from this released arachidonic acid acts as a negative feedback, inhibiting further macrophage production of IL-1, and consequently IL-1 induced IL-2 production (Bonta & Parnham, 1982). IL-2 may reciprocally increase IL-1 production by both stimulated and unstimulated monocytes, and also PGE_2 production by the former (Tilden & Dunlap, 1989).

Production of E-type PGs by guinea-pig peritoneal macrophages was shown to inhibit activation of lymphocytes, isolated from the lymph node, and the secretion of lymphokines in response to antigen. PGE_1 and PGE_2 did not affect the action of preformed lymphokine, indicating the formation rather than the action of the lymphokine on target cells was being inhibited. It was suggested that PGs of the E-series were produced in response to lymphokine stimulation, thereby providing a negative feedback mechanism for the regulation of the cellular immune response (Gordon *et al.*, 1976).

Using a preparation of human peripheral blood mononuclear cells, Goodwin *et al.* (1977b) showed PGE_1 and PGE_2 at 3×10^{-8} M to suppress mitogen stimulation as measured by [^{3}H-thymidine] incorporation. $PGF_{1\alpha}$ and $PGF_{2\alpha}$ had little effect in comparison, whilst PGA inhibited at high concentrations (3×10^{-6} M). The PG synthetase inhibitors indomethacin and RO-20-5720 enhanced mitogen stimulation at non-toxic doses, suggesting an endogenous production of PGs was suppressing the [^{3}H-thymidine] incorporation. This enhancement by indomethacin was greatly reduced when the mononuclear cells were depleted of adherent cells by passing over glass wool columns. These adherent cells must therefore be the PG-producing cells. By removing their inhibitory influence an enhanced mitogen response was expected, but consistent changes were not observed, suggesting a balance between helper and suppressor effects of the adherent monocytes. Overnight pre-incubation of peripheral blood mononuclear cells has been shown to result in a loss of sensitivity to PGE_2 (Goodwin *et al.*, 1978), which may be the result of a loss of receptors. When compared to fresh cells, the pre-incubated cells show an increased response to mitogen, a loss of the enhancing effect of indomethacin inversely related to the dose of mitogen, and no inhibition of the mitogen response to PGE_2. This supports the role for PGs in suppressing mitogen-stimulated lymphocytes *in vitro*.

More recently, PGE_1 and PGE_2 were shown to inhibit production of IL-2 by normal human peripheral blood mononuclear cells (PBMC) at a pathophysiological concentration (10 ng/ml, $\sim 3 \times 10^{-8}$ M), whereas $PGF_{1\alpha}$, $PGF_{2\alpha}$ and PGA_2 did not, PGA_1 inhibiting at 1 mg/ml ($\sim 3 \times 10^{-6}$ M) (Rappaport & Dodge, 1982). Furthermore, the PG synthetase inhibitors indomethacin and fentiazac raised IL-2 levels above basal levels, supporting the inhibitory role for PGE. The mononuclear cell population can be divided into adherent and non-adherent cells. Removal of these adherent cells resulted in an increase in IL-2 production by the non-adherent lymphocytes. In addition there was a loss of sensitivity to the PG synthetase inhibitors, in the absence of any

change in sensitivity to PGE_2. Together, these results suggest PGE_2 produced by the adherent macrophage inhibits the IL-2 production by the lymphocytes, thereby exerting an anti-inflammatory effect.

Experiments carried out on lipopolysacharride (LPS) -stimulated murine resident peritoneal macrophages, indicate that LPS stimulates IL-1 production and arachidonic acid metabolism via the cyclooxygenase pathway, the latter giving rise to PGE_2 and PGI_2. The macrophage cultures produced increased levels of PGE_2 in the presence of exogenous IL-1. Addition of exogenous PGE_2 or PGI_2 resulted in a dose-dependent suppression of LPS-induced macrophage IL-1 production, whilst inhibitors of the cyclooxygenase pathway, indomethacin, piroxicam and ibuprofen, caused a dose-dependent increase, correlating with their potencies as cyclooxygenase inhibitors. Bahl *et al.* (1989) suggested that NSAIDs elevated IL-1 secretion by a mechanism other than inhibition of PGE_2, though the values for PGE_2 represent only duplicate measurements. Arachidonic acid metabolites formed by the cyclooxygenase pathway therefore suppress the production of growth factors by LPS-stimulated macrophages, IL-1 regulating its own production through the self-induced inhibitor, PGE_2 (Kunkel *et al.*, 1986).

Mouse peritoneal macrophages are rich in arachidonic acid, having several times a higher content than other cells (Scott *et al.*, 1980). Indeed, 25 % of the total fatty acid content of this cell is composed of arachidonic acid. Similar levels are thought to be present in both rabbit and human mononuclear phagocytes. Following phagocytosis, 51 % of this is recovered as PGE_2. Human blood monocytes primarily produce TxB_2 and PGE_2 (Dudley & Burns, 1982). These arachidonic acid metabolites participate in inflammation, hypersensitivity and immunoregulation. The release of immunomodulatory prostanoids by inflammatory macrophages adherent to a denatured collagen or fibronectin surface, is significantly more than that when adherent to tissue culture plastic or extracellular matrix

(Gudewicz & Frewin, 1991). This may serve to suppress cytotoxic mechanisms during wound injury.

4.1.4 Are suppressor cells involved in mediating the PGE_2 effect?

Identification of high-affinity binding sites for PGE on human lymphocytes has suggested PGE_2 may also exert a direct inhibitory effect on the lymphocyte itself (Goodwin *et al.*, 1979). Indeed, the lymphocytes themselves produce IL-1 when stimulated by LPS, an effect inhibited by PGE_2 (Herman & Rabson, 1984). Tilden & Balch (1982) addressed the question of whether PGE_2 mediated its inhibition of human peripheral blood mononuclear cell (PBMC) proliferation by a direct inhibition of IL-2 responses, or indirectly by activation of a suppressor cell. Depletion of phenotypically defined suppressor cell populations from PBMC did not diminish the PGE_2-mediated inhibition of the phytohaemaglutinin response. PBMC preincubated with PGE_2 lacked suppressor cell function in the absence of additional PGE_2. They concluded there was no evidence for PGE_2-induced suppressor function in man.

However, inhibition of human lymphocyte IL-2 production by the addition of PGE_2 or monocytes was not observed if the mononuclear cell preparation was pre-irradiated (Chouaib & Fradelizi, 1982). The IL-2 producing cells were not inhibited by the irradiation, and irradiation of monocytes did not affect their inhibitory action. In addition, irradiation enhanced IL-2 production by the mononuclear cell preparation regardless of whether or not it had been depleted of adherent monocytes. This suggests that activation of a radiosensitive lymphocyte by monokines is required for the inhibition of IL-2 production and is supported by the identification of a subset of T-cells that binds PGE_2, some of which may be activated into suppressor cells (Fischer *et al.*, 1985). In another study (Chouaib *et al.*, 1984) it was reported that dialysed supernatants from PGE_2-induced suppressor cells could not reproduce the suppression phenomenon, suggesting the suppressor mechanism requires direct cell to cell contact or a dialysable suppressor factor is

involved. PGE_2 has in fact been reported to promote the secretion of soluble suppressor factors such as prostaglandin-induced T-cell derived suppressor from murine glass adherent splenic T-cells (Rogers *et al.*, 1980,1984).

4.1.5 A PGE-independent effect of precursor fatty acids

The PGE precursors, dihomo-γ-linolenic acid (DGLA) and arachidonic acid (AA), have also been shown to inhibit IL-2 production by human PBMC. This inhibition was only partially blocked by the cyclo-oxygenase inhibitor indomethacin in two of three donors. Also, DGLA and AA inhibited IL-2 production by a cell line insensitive to the inhibitory effect of PGE_2 observed with normal PBMC. It is therefore suggested that the fatty acid precursors to PGE may exert an anti-inflammatory effect by a PGE-independent mechanism (Santoli & Zurier, 1989). A dietary reduction of essential fatty acids (EFA) resulted in a loss of endogenous PG production and enhanced cellular events during chronic inflammation (Bonta & Parnham, 1980). The consequences of this EFA-deficiency could not be fully reversed by exogenous PGs, supporting this notion that PG depletion is not the only cause of the effects observed.

In addition, dietary fatty acids have been shown to alter the number of PGE_2 binding sites, possibly through a change in the bioavailability of PGE_2 from arachidonic acid (Opmeer *et al.*, 1984). The study involved peritoneal macrophages isolated from rats pretreated for 4-weeks with a diet containing 12.5 % corn oil, in which an increase in the total number of high and low affinity PGE_2 binding sites in the absence of any alteration in the K_d values was shown. This was followed by a parallel leftward shift in the dose-response curve of PGE_2-stimulated adenylate cyclase. It was suggested that the linoleic acid present in the corn oil may have reduced the amount of arachidonic acid released, and consequently the level of the arachidonic acid metabolite PGE_2. The observed increase in receptor number may represent a protective mechanism to overcome insufficient stimulation.

Alteration of dietary fatty acids may therefore be of important use in the treatment of inflammatory disorders.

4.1.6 HL-60 cells differentiate into human macrophage-like cells

Since IL-1 and IL-2 are immunomodulatory and inflammatory, PGE_2 may exert an anti-inflammatory effect through inhibition of their release, as described. It is therefore of interest to characterise the subtype of PGE receptor present on the macrophage, in order to understand the molecular basis of the inhibitory activity of PGE_2 in regulating macrophage function. I have used radioligand binding techniques on a purified plasma membrane preparation from differentiated HL-60 cells, which differentiate into human macrophage-like cells on exposure to phorbol ester.

Cloning of bone marrow stem cells has indicated a common stem cell gives rise to both monocytes and granulocytes. The promyelocytic cell line, HL-60, is derived from a 36 year-old Caucasian woman with acute promyelocytic leukaemia (Collins *et al.*, 1977) and provides a useful tool in studying the cellular and molecular events involved in proliferation and differentiation of normal and leukaemic cells of the granulocyte or macrophage lineage (Collins, 1987). The cell line exists in an arrested yet pliant state of maturation.

Spontaneous differentiation beyond the promyelocytic stage may occur in up to 10 % of the cultured cells. However, differentiation into macrophages or granulocytes can occur under the influence of a series of compounds. Dimethylsulphoxide, hypoxanthine, dimethyl formamide, actinomycin D, and retinoic acid all induce differentiation to granulocytes. Differentiation to macrophages occurs under the influence of phorbol ester, 1,25-dihydroxy Vitamin D_3, sodium butyrate, dibutyryl cyclic AMP, and agents which elevate cyclic AMP, such as prostaglandin E_2.

In order to determine if the HL-60 population represents

homogeneous bipotent stem cells or a mixture of cells committed to either myeloid or monocytoid differentiation, a cloned population of HL-60 cells has been isolated and studied. The experiments involved the use of dimethylformamide (DMF) and TPA which induce myeloid and monocytoid differentiation respectively. Besides inducing differentiation of this cloned population of cells along two distinct lines, TPA is reported to reduce the percentage of cells exhibiting the myeloid phenotype in response to DMF, while increasing that with the macrophage phenotype (Fontana *et al.*, 1981).

HL-60 cells grown in suspension culture terminally differentiate into adherent human macrophage-like cells in the presence of phorbol ester (Rovera *et al.*, 1979a,b), of which 12-o-tetradecanoylphorbol 13-acetate (TPA) is the most active. The evidence is based on the appearance of markers in TPA-treated HL-60 cells that are specific to the monocyte / macrophage lineage or the disappearance of myeloid markers. The undifferentiated HL-60 cells contain a large nucleus with diffuse chromatin and a prominent nucleolus. The Golgi apparatus is well defined and associated with a variable number of vacuoles, many of which have a dense core. Whilst lacking specific markers for lymphoid cells, the HL-60 cells express surface receptors for the Fc fragment of IgG and complement. They are, however, unable to specifically ingest IgG-coated erythrocytes. HL-60 cells respond to chemotactic stimuli (Gallagher *et al.*, 1979).

Following differentiation, the TPA-treated cells become adherent and develop pseudopodia. The nucleus is round or reniform with a prominent nucleolus and the chromatin is condensed. Cell proliferation is arrested but the cells are still able to synthesise protein. In contrast to the undifferentiated form, the Golgi apparatus is associated with small granules of uniform density, and the extent of the rough endoplasmic reticulum and number of mitochondria are increased. Fc-IgG receptors are present as determined by fluorescent antibody studies. The differentiated HL-60 cells exhibit phagocytic and microbicidal activity, but not chemotaxis (Koeffler *et al.*, 1981). Cellular levels of NADase, acid

phosphatase, and non-specific esterase, enzymes reported to be characteristic markers of monocytes and macrophages, are markedly increased after treatment with TPA. Myeloperoxidase activity is reduced, as would be expected since it is a myeloid marker. More lysozyme is also found in the medium of TPA-treated cells than in the medium of untreated cells. All of these features closely resemble those of the human monocyte or macrophage.

There are however limitations to the use of cell culture as a substitute for the normal human macrophage itself. Mononuclear phagocytes for instance should display chemotactic activity, yet the differentiated HL-60 cells do not. However, a low level of chemotactic activity has been demonstrated for macrophages and monocytes in culture, and the lack of activity in the TPA-treated cells may therefore be a consequence of the environment in which they are maintained. The TPA-treated HL-60 cells also lack the large digestive vacuoles or secondary lysosomes present in mature stimulated macrophages. Newburger *et al.* (1981) report that whilst TPA-induced HL-60 cells develop the morphological appearance and enzymatic characteristics of macrophages, they lack the functional capacities of the mature phagocytic cells.

4.1.7 Mechanism of TPA-induced differentiation of HL-60 cells

TPA is a tumour promoter (Diamond *et al.*, 1980) and protein kinase C (PKC) activator (Castagna *et al.*, 1982), with which it copurifies (Vandenbark *et al.*, 1984). PKC is a calcium- and phospholipid-dependent enzyme that is widely distributed in numerous tissues, and which plays a crucial role in transducing various extracellular signals across the cell membrane. In platelets (Drummond, 1987), endogenously produced diacylglycerol (DAG) activates PKC, increasing its affinity for calcium and phospholipid. TPA can substitute for DAG, activating PKC without the need for PI turnover, from which DAG is derived.

The mechanism by which TPA induces growth arrest and

differentiation is not clear. This activation of PKC in HL-60 cells could result in the phosphorylation of specific substrate proteins leading to the macrophage-like phenotypic changes induced by TPA. Studies using chick embryo fibroblasts suggest that the particulate preparation is able to specifically bind phorbol esters at concentrations known to induce macrophage differentiation (Driedger & Blumberg, 1980), indicating the presence of a receptor. The interaction of the ligand with its receptor is rapid, fully reversible and of high affinity, with a K_d in the nanomolar range. Phorbol esters are also reported to exert their effects while retained at the cell surface of HL-60 cells (Cooper *et al.*, 1982). The high lipophilicity of TPA (Jacobson *et al.*, 1975) causes it to partition strongly into membranes. The amphipathic phorbol esters may then bind nonspecifically, changing the structure and function of the plasma membrane and thereby inducing the observed differentiation. The low concentration at which it is effective, however, suggests it may laterally diffuse through biomembranes to distant receptors after binding to the bilayer portions of the membrane.

The ability of these leukaemic cells to undergo maturation in the presence of certain inducers, indicates that the genetic information coding for terminal macrophage differentiation is present and capable of expression. There is a change in the relative abundance of certain translational products in HL-60 cells on exposure to TPA, indicating a change in the levels of different mRNA or the ability of mRNA to be translated, suggesting TPA-induced signals received at the cell membrane are conveyed to the nucleus. The changes recorded are not the same as those following myeloid differentiation (Colbert *et al.*, 1983). Nuclear proto-oncogenes such as c-fos, c-jun and c-myc are examples of immediate early genes expressed during the early phase of responses leading to growth arrest or stimulation (Shimizu *et al.*, 1991). TPA can initiate rapid induction of c-fos mRNA and protein during myelomonocytic differentiation and macrophage differentiation (Muller *et al.*, 1983) and is also reported to induce c-jun expression in human myeloid leukaemia

cells (Sherman *et al.*, 1990). Macrophage-like differentiation of HL-60 cells is associated with a marked decrease in c-myc RNA levels. Recent evidence indicates the expression of a novel immediate early gene during the TPA-induced macrophagic differentiation of HL-60 cells (Shimizu *et al.*, 1991). The protein derived from this novel gene may be a nuclear DNA-binding protein which regulates transcription. The pathway leading to induction of this gene is inhibited by the PKC inhibitor H7 and stimulated by the PKC activator 1,2-dioctanoylglycerol (diC8). The protein phosphatase inhibitor okadaic acid also enhances expression. Together these results suggest the pathway of induction of the early gene by TPA to be via PKC, and to be controlled by protein phosphorylation.

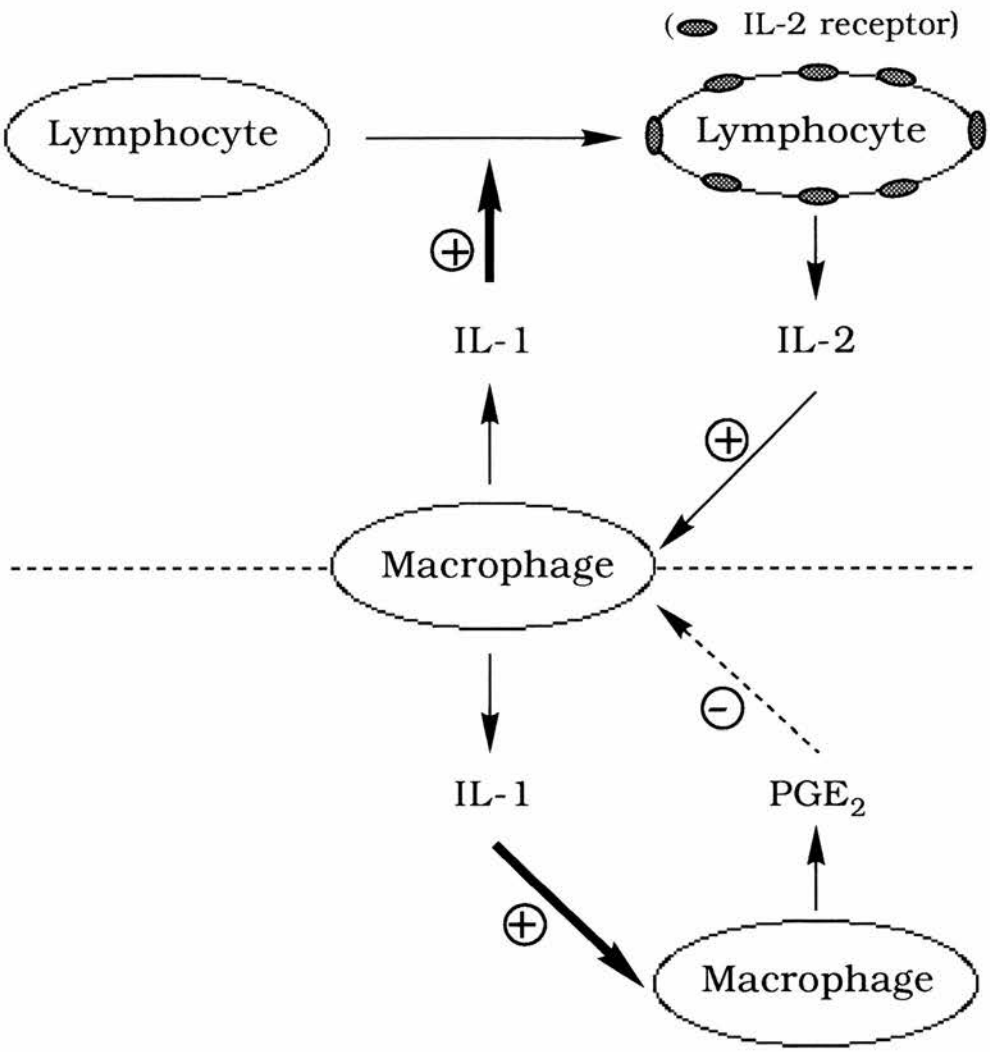
However, activation of protein kinase C is not thought to be the only mechanism by which phorbol esters induce differentiation of human promyelocytic cells to macrophage-like cells. Kreutter *et al.* (1985) used TPA and 1-oleoyl-2-acetylglycerol (OAG) as activators of PKC. At similar biochemical activities, OAG failed to induce HL-60 differentiation. The profile of protein phosphorylation is more extensive in the presence of TPA as compared to OAG. This may reflect the activation of a kinase other than PKC, since both TPA and OAG activate PKC to the same extent. However, the same group later reported (Morin *et al.*, 1987) that if HL-60 cells are exposed concurrently to a cell-permeable diacylglycerol, diC8, and the calcium ionophore A23187, at concentrations without maturational activity when used separately, there are measurable increases in both protein phosphorylation and the fraction of cells expressing the differentiated phenotype. This suggests that there are specific biochemical effects associated with TPA, in addition to the activation of PKC, which may be important determinants for the induction of leukaemia cell differentiation. These may include an increased cellular permeability to calcium or mobilisation of intracellular pools, in the light of the results obtained in the presence of A23187.

The activation of PKC by TPA may be secondary to a stimulated

increase in phospholipid turnover. This would result in an increase in metabolism of DAG, an increase in the release of arachidonic acid which itself activates PKC, and generation of specific eicosanoids which might promote or induce leukaemia cell differentiation. Humes *et al.* (1978) report on the stimulation of release of arachidonic acid and its cyclooxygenase products from macrophages by TPA. It has been suggested that arachidonic acid may also be involved in the mechanism of action of TPA (Das, 1991). Phorbol ester is concentrated largely in the lipid phase of the cell membranes, and an early response to it is the release from the membrane phospholipids of arachidonic acid (Levine, 1981). Indeed an increase in cyclo-oxygenase activity, as a result of newly synthesised enzyme, has been reported for both monocytic and granulocytic differentiation of HL-60 cells (Honda *et al.*, 1990). Sanduja *et al.* (1988) suggest selective metabolites rather than the entire cyclooxygenase pathway may be increased in an inducer-specific manner, though Dudley & Burns (1982) propose an inability of HL-60 derived macrophages to directly utilise exogenous arachidonic acid or mobilise endogenous subsrate for production of PGs.

Whilst the understanding of the mechanism of differentiation of HL-60 cells remains incomplete, this cell-line allows for a constant supply of macrophage-like cells in a controlled manner and in considerably greater numbers than if isolated from human blood.

(a) *macrophage-lymphocyte interaction*



(b) *macrophage-macrophage interaction*

Figure 4.1.1 Interaction between mononuclear cells

4.2 MATERIALS and METHODS

SOLUTIONS		Source
Tris - HCl 500 mM pH 7 .4		
121.14 g	tris(hydroxymethyl)methylamine	B.D.H. Chemicals
~ 40 ml	concentrated HCl	"
- made up to 2 l with distilled water.		
Tris - HCl 50 mM pH 7.4		
100 ml	tris - HCl (500 mM pH 7.4)	
adjust pH to 7.4 with concentrated HCl		
- made up to 1 litre with distilled water.		
Homogenisation buffer		
50 ml	tris - HCl (50 mM pH 7.4)	
42.8 g	sucrose	"
1 mM	indomethacin	Sigma (I 7378)
- made up to 500 ml with distilled water.		
Sucrose		
0.9 M	30 g sucrose	B.D.H. Chemicals
1.2 M	40 g sucrose	"
- in 100 ml tris - HCl 50 mM pH 7.4.		
250 mM Magnesium chloride		
5.1 g	$MgCl_2 \cdot 6H_2O$	"
- in 100 ml tris - HCl 50 mM pH 7.4.		

SOLUTIONS *continued* Source

0.5 mg / ml Bovine serum albumin

5 mg BSA fraction V B.D.H. Chemicals
- in 10 ml tris - HCl 50 mM pH 7.4

or

1 ml 2 mg / ml BSA standard Pierce (No. 23209)
to 3 ml tris - HCl 50 mM pH 7.4.

0.1 M Sodium hydroxide

4 g NaOH B.D.H. Chemicals
in 1 l distilled water.

2 % Sodium carbonate in 0.1 M NaOH

2 g Na_2CO_3 "
- in 100 ml 0.1 M NaOH.

2 % Potassium sodium tartrate

0.2 g $C_4H_4O_6KNa.4H_2O$ "
- in 10 ml tris - HCl 50 mM pH 7.4.

1 % Copper sulphate

0.1 g $CuSO_4.5H_2O$ "
- in 10 ml tris - HCl 50 mM pH 7.4.

50 % v / v Folin & Ciocalteu's phenol reagent

10 ml Folin-Ciocalteu reagent "
- in 10 ml tris - HCl 50 mM pH 7.4.

CHEMICALS	Source
AH 6809	Dr. R.A. Coleman, Glaxo, U.K.
GR 32191	"
butaprost	Dr. P. Gardiner, Bayer, U.K.
cicaprost	Prof. H. Vorbruggen, Schering AG, Berlin
sulprostone	"
nat 11-deoxy PGE_1	Cayman Chemicals, U.S.A.
PGE_2	"
[5,6,8,11,12,14,15 - ^3H (N)] PGE_2	NEN (NET-428)
12-o-tetradecanoylphorbol 13-acetate	Sigma (P 8139)

CELL CULTURE REAGENTS	Source
RPMI 1640 with L-glutamine / Glutamax	Gibco (041-01 / 31 870)
Penicillin - Streptomycin 10,000 IU/ml; 10,000 µg/ml	" (043-05140)
Heat - inactivated foetal calf serum	" (031-6290)
Hanks' balanced salt solution (HBSS) (without calcium and magnesium)	" (041-04170)

4.2.1 Passing of HL-60 cells

HL-60 cells were obtained from the European Collection of Animal Cell Cultures. All procedures required sterile technique.

The growth medium contained :

RPMI 1640 with 2 mM L-glutamine or glutamax
10 % foetal calf serum (FCS)
100 IU/ml penicillin
100 μg/ml streptomycin

and may be stored at 4 °C for a maximium of one week.

The cells in suspension were pelleted by centrifugation at 300 g and room temperature for 10 min. Once suspended in 50 ml fresh growth medium in 175 cm^2 plastic flasks, they were incubated at 37 °C in a humidified 5 % CO_2 atmosphere. When seeded at a concentration of 2 x 10^5 cells / ml, the doubling time was approximately 1-2 days. Cell numbers were counted using a haemocytometer, trypan blue exclusion determining viability. The RPMI medium contained the indicator phenol red, and the cells were passed before this was allowed to turn yellow.

4.2.2 Plasma membrane preparation from differentiated HL-60 cells

The method for the preparation of the plasma membrane - rich fraction in differentiated HL-60 cells, has been developed from procedures for the preparation of a similar fraction from Swiss 3T3 cells (Allergan Pharmaceuticals) and the murine macrophage-like cell line $P388D_1$ (Fernandez-Botran, 1984 a,b).

1. 20-30 hr before preparation of the membranes, 16 nM TPA (in acetone) was added to each of 15 cell culture flasks, containing on average 27 ± 2 x 10^6 cells. An equivalent concentration of acetone alone did not induce adherence and the HL-60 cells remained viable as measured by trypan blue exclusion.

2. When differentiated the cells became adherent, the culture medium was removed by aspiration and the cells washed once with 10 ml / flask HBSS to ensure the removal of non-viable undifferentiated HL-60 cells. The differentiated cells were harvested by scraping into HBSS.

3. Following centrifugation at 300 g and 4 °C for 10 min, the resultant pellet was washed once again in HBSS, and then in homogenisation buffer. Whilst the 5 mM tris-HCl promotes bursting of the cell membrane, 250 mM sucrose mimics the cytoplasmic tonicity to which intracellular organelles are exposed in order to avoid swelling and even possible disruption. Indomethacin minimises unwanted occupancy of binding sites by endogenously synthesised PGs.

4. The pellet was resuspended in 40 ml homogenisation buffer and homogenised for 3 sec at setting 7 on the polytron homogeniser (model X 1020).

5. After centrifugation at 300 g and 4 °C for 5 min, the supernatant was removed to a clean tube.

6. The remaining pellet was once again suspended in 25 ml homogenisation buffer, rehomogenised and centrifuged, and the supernatant pooled with that obtained previously. The crude homogenate preparation may be stored at -70 °C at this stage and combined with further preparations before completing the remainder of the plasma membrane preparation. In such a case, the binding study was carried out without refreezing the membrane fraction.

7. The combined supernatants were centrifuged at 177,000 g and 4 °C for 40 min in a Kontron ultracentrifuge (Centrikon T-2070) using a Typ TFT 50.38 (12287) fixed-angle rotor.

8. The pellet was resuspended in 48 ml homogenisation buffer using setting 5 on the polytron for 1 or 2 min until the pellet was well dispersed.

9. This crude homogenate preparation was layered onto a sucrose density gradient in 6 tubes, 8 ml layered over 3 ml each of 0.9 M over 1.2 M sucrose. These were then centrifuged at 112,000 g and 4 °C for 2 h in an MSE Superspeed 65 ultracentrifuge, using the swing-out rotor 43127-506.

10. The band at the interface of the 0.9 M / 1.2 M sucrose was removed, diluted approximately 1:2 with 50 mM tris-HCl pH 7.4 and centrifuged at 304,000 g and 4 °C for 40 min.

11. The pellet was washed once with 50 mM Tris-HCl pH 7.4, and if not frozen at the crude homogenate stage, stored at -70 °C for future binding studies. The protein recovered in three of these membrane preparations was sufficient for two displacement studies, each covering 13 concentrations of competing ligand tested in triplicate.

4.2.3 Binding studies using the purified plasma membrane fraction.

1. The standard binding medium consisted of 2.5 mM $MgCl_2$ in 50 mM tris-HCl pH 7.4. The plasma membrane pellet was resuspended in 50 mM tris-HCl pH 7.4 by sonication or glass homogenisation. Binding reactions were initiated by the addition of 100 ml of the plasma membrane fraction (approximately 30 μg of protein), to a reaction mixture containing [^{3}H]-PGE_2 with or without competing ligand, to a final volume of 200 μl. Non-specific binding was determined in each experiment by the presence of 10 μM PGE_2.

2. The samples were mixed and following incubation for 1 h at room temperature, the reaction was stopped by the addition of ice-cold buffer. The plasma membranes were immediately collected on Whatman glass microfibre filters under vacuum using a Brandel cell harvester (model M-24R), and washed twice with the ice-cold buffer.

3. The filters were air dried, placed in scintillation vials containing 5 ml Packard scintillation cocktail and counted on a Packard liquid scintillation analyser 1900CA.

4. Protein content was measured initially according to the method of Lowry (1951) (see below), and then Bradford (1976) using Pierce Coomassie Protein Assay Reagent (No. 23200). (At Allergan I used the Bio-Rad protein assay concentrate (Cat. No. 500-0006) based on the Bradford method.)

4.2.4 *Determination of protein content by the Lowry method.*

1. A standard curve was prepared using the volumes of 0.5 mg / ml BSA and tris-HCl 50 mM pH 7.4 detailed below. 0.5 ml of each unknown was aliquoted out. Samples were assayed in duplicate.

Tube	Volume of BSA 0.5 mg/ml (µl)	Volume of tris-HCl (µl)	Amount of protein (µg)
1 , 2	-	500	-
3 , 4	50	450	25
5 , 6	125	375	62.5
7 , 8	250	250	125
9 , 10	375	125	187.5
11 , 12	500	-	250

2. Reagent A was prepared as follow :

75 ml 2 % Na_2CO_3 in 0.1 M NaOH
+ 0.75 ml 2 % potassium sodium tartrate, mix,
+ 0.75 ml 1 % $CuSO_4$, mix;

50 % Folin-Ciocalteu's reagent was reagent B.

3. At t=0, 2 ml of reagent A was added to each tube at 20 sec intervals and mixed.

4. At t=10 min, 0.25 ml of reagent B was added to each tube at 20 sec intervals and mixed.

5. At t=40 min, the absorbance at 660 nm was measured, which is proportional to the total amount of protein and therefore allows for the determination of the unknown protein content in the plasma membrane samples from the standards.

4.3 LIGAND BINDING THEORY

R - receptor
L - ligand
RL - receptor-ligand complex
k_{+1} - association rate constant
k_{-1} - dissociation rate constant
K_d - equilibrium dissociation constant for ligand
B_{max} - total number of binding sites
b - bound ligand [RL]
F - free ligand [L]
K_i - dissociation constant for an inhibitor
IC_{50} - concentration of inhibitor which gives 50 % inhibition of binding of the ligand

$$[R] + [L] \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [RL]$$

at equilibrium $k_{+1}\,[R]\,[L] = k_{-1}\,[RL]$

therefore $K_d = k_{-1} / k_{+1} = [R]\,[L] / [RL]$

since $[RL] + [R] = B_{max}$

then $[RL]\,[L] + [R]\,[L] = B_{max}\,[L]$

substituting $[RL]\,[L] + K_d\,[RL] = B_{max}\,[L]$

rearranging $[RL] = B_{max}\,[L] / [L] + K_d$

$b = B_{max}\,F / F + K_d$

When $b = B_{max} / 2$

then $B_{max} / 2 = B_{max} F / F + K_d$

therefore $K_d = F$

COMPETITIVE INHIBITORS OF BINDING

Displacement studies involve the use of one [^{3}H] - ligand concentration with increasing concentrations of competing ligand. The relationship is described by the Cheng - Prusoff (1973) equation :

$$K_i = IC_{50} / (1 + [^3H\text{-}L] / K_d)$$

By plotting % of control binding versus log [competing ligand], a value for IC_{50} may be obtained, and consequently K_i determined.

When the [^{3}H] - ligand and displacing ligand display the same physical and chemical properties in the interaction with the binding sites,

$$K_d = K_i$$

and $$IC_{50} = K_d + [L]$$

4.4 RESULTS and development of the procedure

Initial binding studies indicated a greater specific binding in the purified plasma membrane fraction of differentiated HL-60 cells as compared to the crude homogenate (Table 4.1). The higher protein concentration of the crude homogenate resulted in a greater dpm for both total and non - specific binding, though the dpm for specific binding was comparable to that in the purified fraction. The sucrose density - gradient centrifugation step therefore results in a 3-fold increase in the amount of [^{3}H]-PGE_2 bound, measured as fmoles / mg protein.

Table 4.1. Comparison of [^{3}H]-PGE_2 binding to crude homogenate and the purified plasma membrane fraction of differentiated HL-60 cells.

parameter	crude homogenate (n=8)	plasma membrane (n=8)
total binding (DPM)	9077 ± 4481	5191 ± 2013
non-specific binding (DPM)	7419 ± 3729	3821 ± 1731
specific binding (DPM)	1658 ± 765	1370 ± 453
% specific binding of total	19 ± 4	33 ± 4
bound (fmoles/mg)	30 ± 13	105 ± 31

$MgCl_2$ is required for the activation of ATP and therefore adenylate cyclase activity which converts ATP to cyclic AMP. Since PGE_2 stimulates the production of cyclic AMP in macrophages (Bonta & Parnham, 1982) and has been shown to increase cyclic AMP levels in both differentiated and undifferentiated HL-60 cells (see Discussion), the effect of $MgCl_2$ on PGE_2 binding was tested. Specific binding was found to fall significantly in its absence from 43 % to 20 % at 1 nM [^{3}H]-PGE_2, and 37 % to 6 % at 10 nM [^{3}H]-PGE_2. In an experiment in which specific binding was 58 % in the presence of 2.5 mM $MgCl_2$, a fall in specific binding of 30 %

was observed using 0.25 mM $MgCl_2$, whereas 25 mM showed no improvement.

The incubation period for binding was 1 hr at room temperature. An individual experiment showed the binding to rise from 25 % through 36 %, 38 % to 50 % specific binding for 1 nM [^{3}H]-PGE_2 at incubation periods of 15 min, 30 min, 45 min and 60 min respectively. The half-life ($t_{1/2}$) for onset of PGE_2 binding was therefore estimated at 20 min. Considering the temperature of incubation, two experiments demonstrated that increasing the temperature to 37 °C did not improve the % specific binding obtained with a 1 hr incubation at 0.1, 1 or 10 nM [^{3}H]-PGE_2. It was decided to incubate at room temperature since the off-rate is less than at 37 °C, thereby reducing a component of error in separating bound and free radioligand.

As reported in the context of the $MgCl_2$ experiments, PGE_2 stimulates adenylate cyclase and binding sites should therefore exist on the plasma membrane, in which adenylate cyclase is embedded. Following the sucrose gradient centrifugation step to purify the crude homogenate, the interface at which the greatest specific binding was obtained was then taken to represent the plasma membrane - rich fraction. These results are illustrated in Table 4.2.

Table 4.2. Comparison of 2 nM [^{3}H]-PGE_2 binding to various interfaces in the sucrose gradient.

bound (fmoles/mg)	sucrose surface (M)					
	0.9	1.06	1.2	1.3	1.6	1.9
Expt. 1			169	26	13	
Expt. 2			97	32	25	28
Expt. 3	5	50				
Expt. 4	5		33			
Expt. 5		31	48			

The results suggested the band on top of the 1.2 M sucrose layer to have the greatest degree of binding. Since no appreciable specific binding was obtained over the 0.9 M layer, this protein fraction may contribute to non - specific binding, therefore a gradient of 0.9 M over 1.2 M sucrose was used to prepare the purified plasma membrane fraction.

The development of this protocol for the preparation of a plasma membrane - rich fraction allowed subsequent saturation and displacement studies to be carried out in order to characterise the subtype(s) of PGE receptor present on the human macrophage-like cell. The vertical bars on the figures represent the standard error of the mean. The statistical significance ($p<0.05$) of the observed inhibitions of specific [^{3}H]-PGE_2 binding was assessed using the paired Student's t-test. The data have been analysed according to the theory already detailed (pages 145-146) and in addition by the computer programs Totalfit and Fit (Barlow, 1983). The latter analysis was provided by Dr R. Barlow, formerly Reader in Chemical Pharmacology at the University of Bristol, to whom I am most grateful.

Experiments carried out at Allergan Pharmaceuticals involved determinations of the specific binding of [^{3}H]-PGE_2 over the concentrations range 0.1 nM to 100 nM. The results are represented as bound *vs* free (Figure 4.1(a),(b)), and suggest the presence of two binding sites, a saturable high affinity site and a lower affinity site which is not saturated at the highest radioligand concentration used. Considering only those data points to 50 nM (Figure 4.1(b)), at which concentration the higher affinity site appears to be saturated, the average K_d, determined from B_{max} / 2 for individual experiments, is given by 2.5 ± 0.4 nM. Using a program based on Totalfit, this same data has been fitted to a 'binding process + chemical reaction' for those sets of data covering the full range of concentrations (n=5), and to mass action binding for those limited to the first saturated site (n=2). The value of K_d is determined in each case, and the mean (n=7) is given by 7.3 ± 2.6 nM.

Displacement of 2 nM [^{3}H]-PGE_2 by PGE_2 itself (n=4-5) revealed an IC_{50} of 17 ± 1 nM, corresponding to a K_d of 15 ± 1 nM (Figure 4.2). 11-deoxy PGE_1 also displaced specifically bound [^{3}H]-PGE_2 with an IC_{50} of 30 ± 17 nM (Figure 4.2), which may be transformed into a K_i of 27 ± 15 nM. Surprisingly, whilst PGE_2 and 11-deoxy PGE_1 achieved significance ($p<0.01$) at concentrations equal to and above 10^{-8} M and $5x10^{-7}$ M respectively, butaprost only significantly displaced [^{3}H]-PGE_2 to this extent at 10^{-5} M and AH 13205 did not achieve significance at concentrations up to 10^{-5} M (Figure 4.3). In a similar manner to butaprost, sulprostone significantly displaced [^{3}H]-PGE_2 ($p<0.01$) only at 10^{-5} M, at which concentration AH 6809 also achieved significance though to a lesser degree ($p<0.05$) (Figure 4.4). Interestingly the IP-receptor agonist cicaprost displaced specific binding with an IC_{50} of 4.8 ± 1 μM, K_i of 4.2 ± 0.6 μM, (n=3-4), significant ($p<0.01$) at 10^{-5} M (Figure 4.5).

This displacement data has been analysed using the logistic fit, developed from Fit, and with fit to two sites, developed from 2SiteInhib in Biodata handling (Barlow, 1983). In the self displacement studies one binding site is revealed with mean K_d given by 26 ± 5 nM. This contrasts with the two binding sites obtained in three out of four experiments where 11-deoxy PGE_1 displaced specifically bound [^{3}H]-PGE_2. The mean results for these experiments are K_{i1} = 12 ± 2 nM (n=3); 23 ± 11 nM (n=4) and K_{i2} = 3121 ± 87 nM. The maximum effect of the first component of this binding is approximately 10 times that of the second.

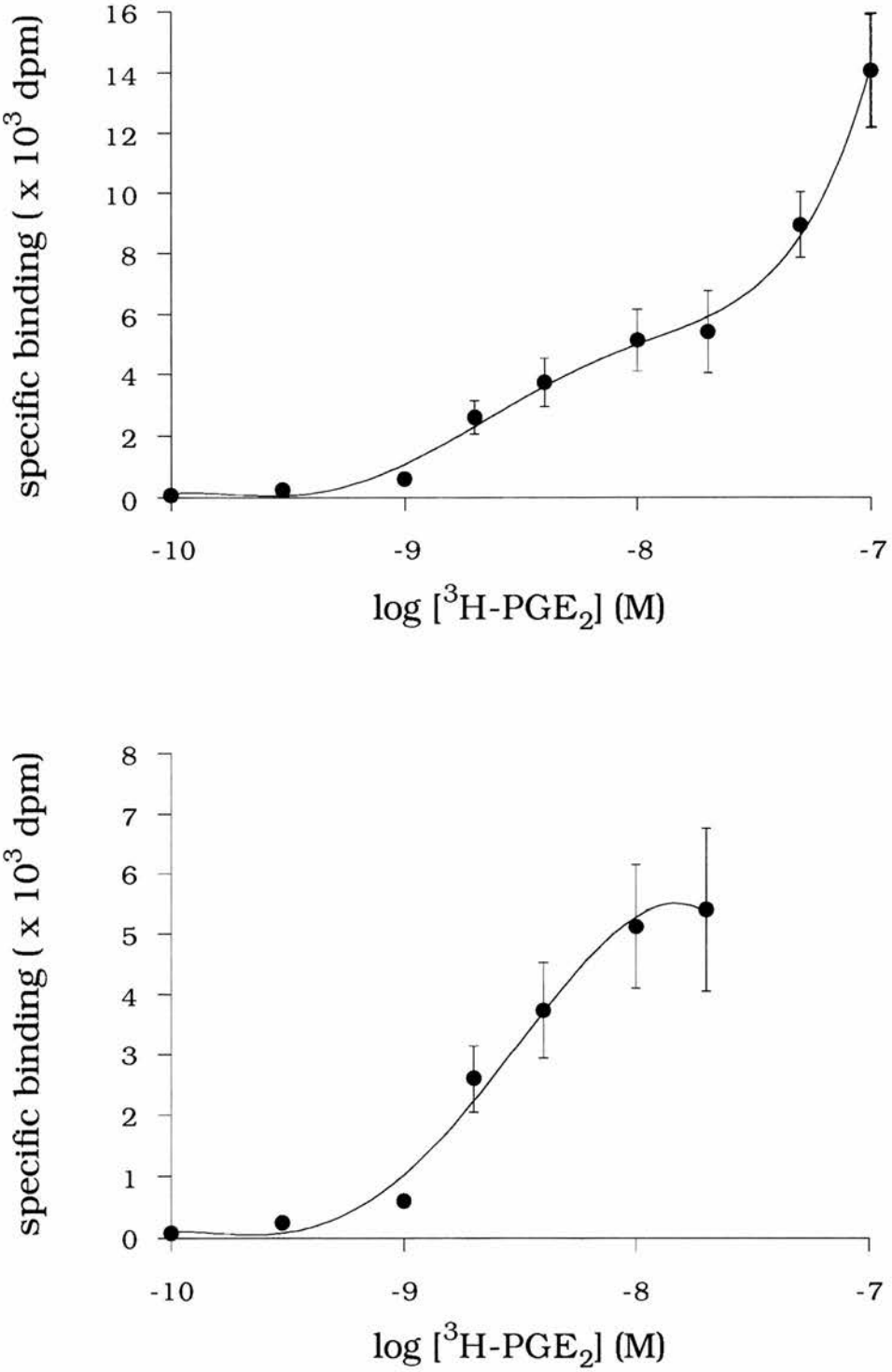
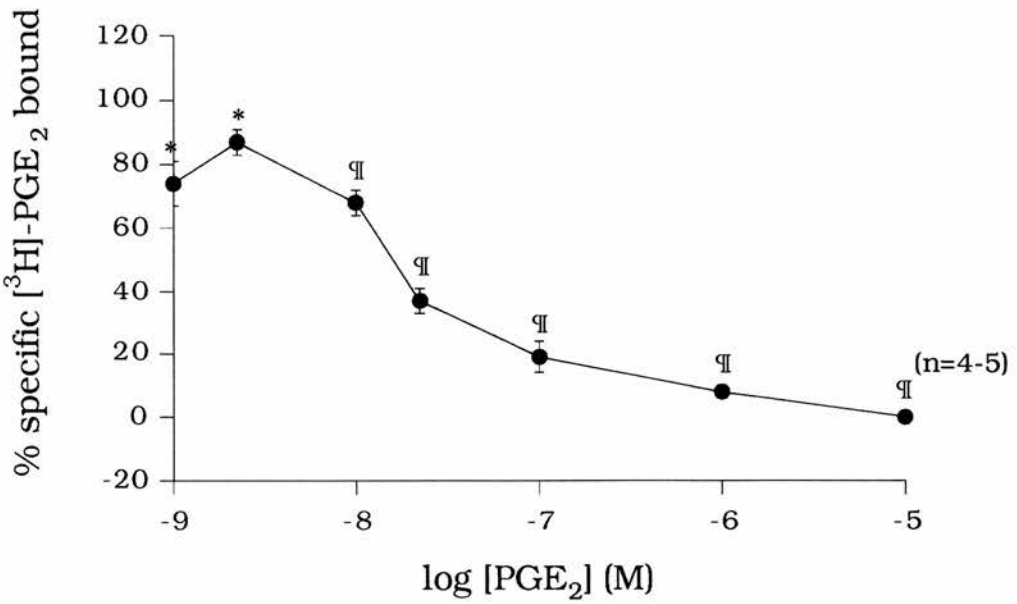


Figure 4.1 Saturation experiment using increasing concentrations of [^{3}H]-PGE$_2$ (n=5-7), illustrating
(a) two binding sites;
(b) the saturable high affinity site.
(Curves constructed using polynomial curve fit application in Kaleidagraph, Apple Macintosh.)



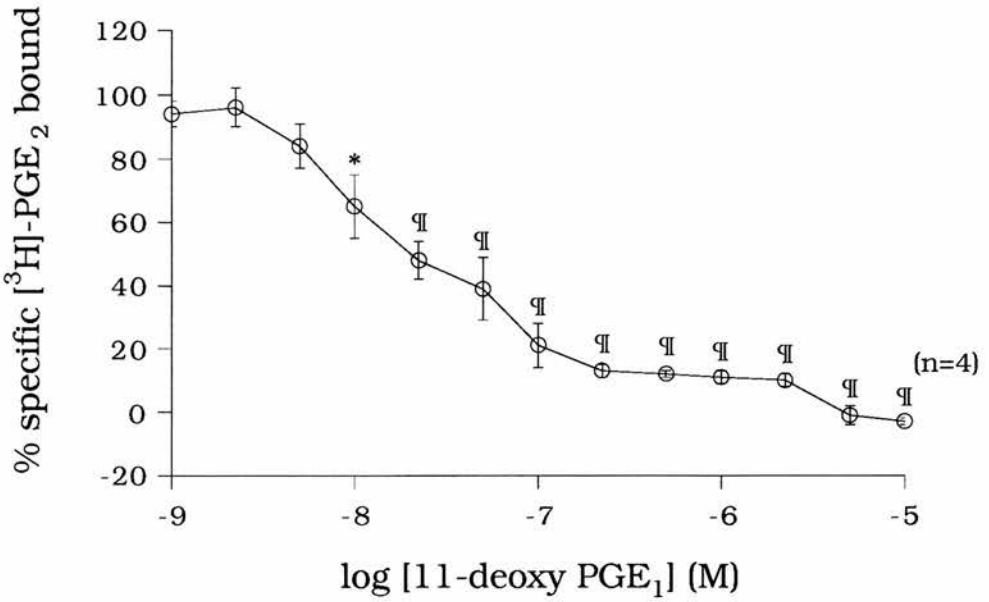


Figure 4.2 Displacement of 2 nM [^{3}H]-PGE_2 from the human macrophage-like plasma membranes by PGE_2 and the EP_2 - selective agonist, 11-deoxy PGE_1.
* ($p<0.05$) and ¶ ($p<0.01$) denote significant displacement of specific [^{3}H]-PGE_2 bound.

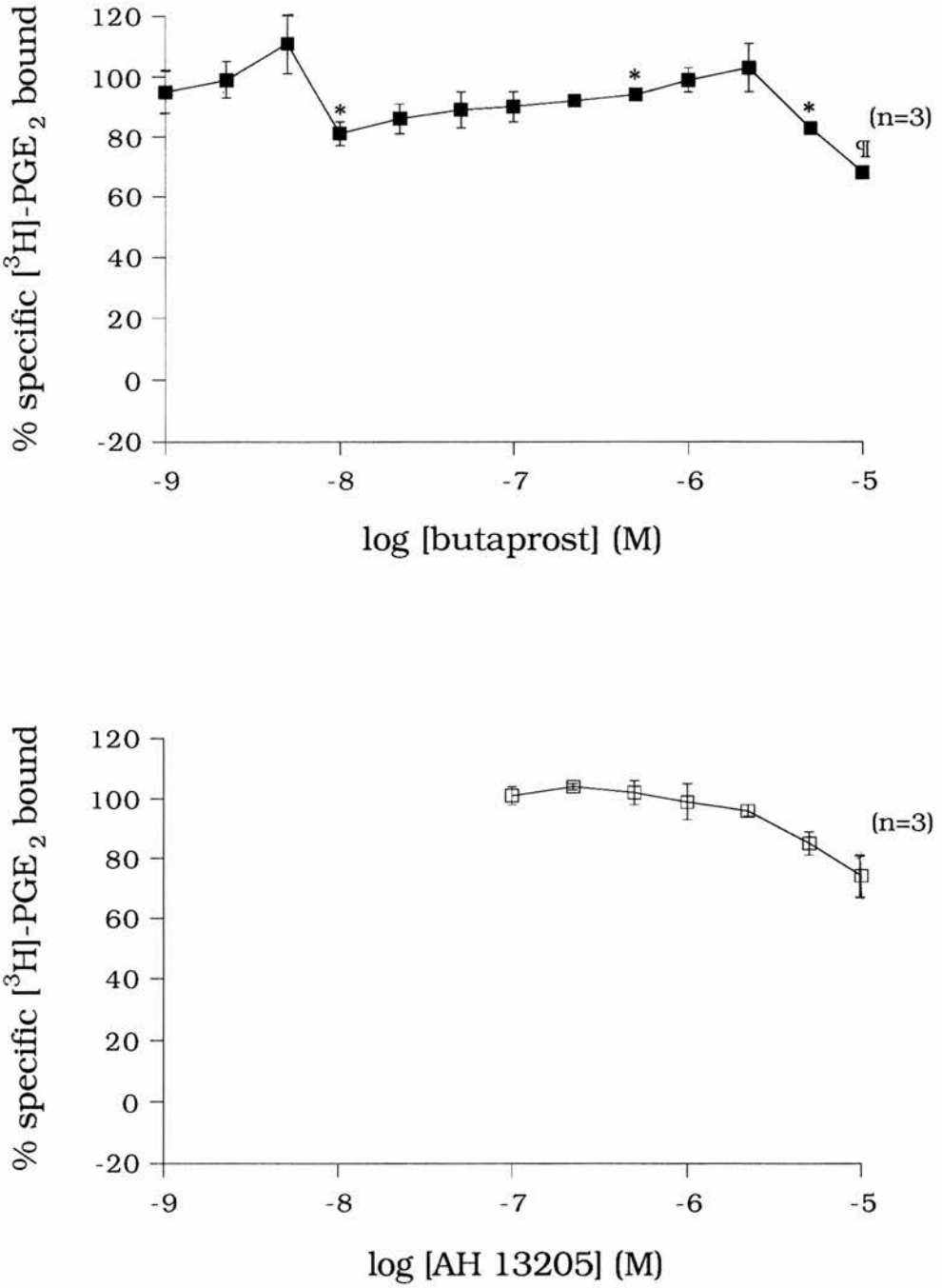


Figure 4.3 Displacement of 2 nM [^{3}H]-PGE_2 from the macrophage-like plasma membranes by the EP_2 - selective agonists, butaprost and AH 13205.
* ($p<0.05$) and ¶ ($p<0.01$) denote significant displacement of specific [^{3}H]-PGE_2 bound.

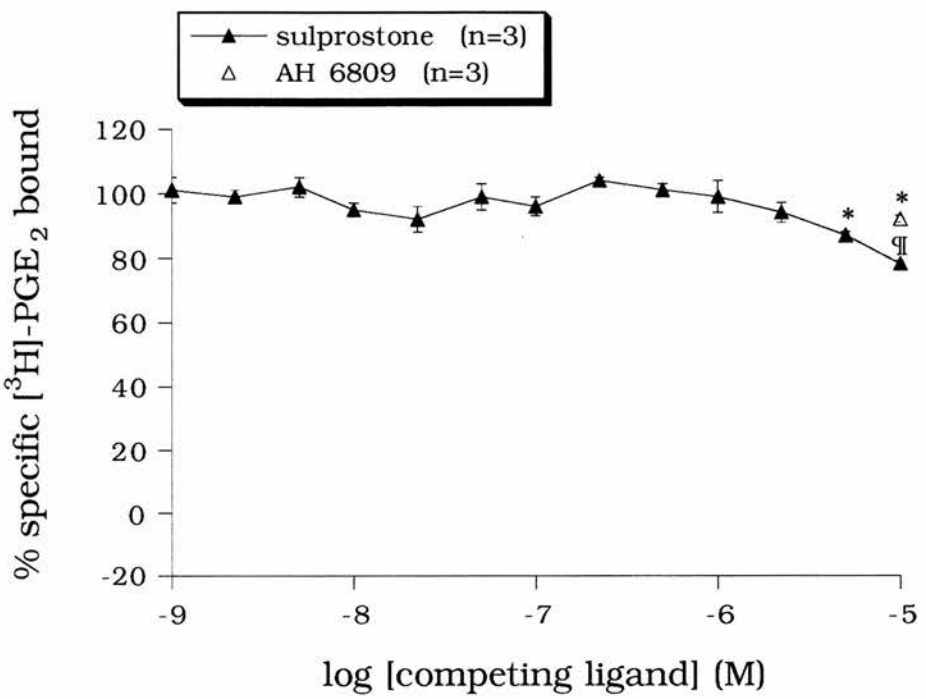


Figure 4.4 Displacement of 2 nM [^{3}H]-PGE_2 from human macrophage-like plasma membranes by the EP_1 - & EP_3 - selective agonist sulprostone, and the EP_1 - selective antagonist AH 6809.
* ($p<0.05$) and ¶ ($p<0.01$) denote significant displacement of specific [^{3}H]-PGE_2 bound.

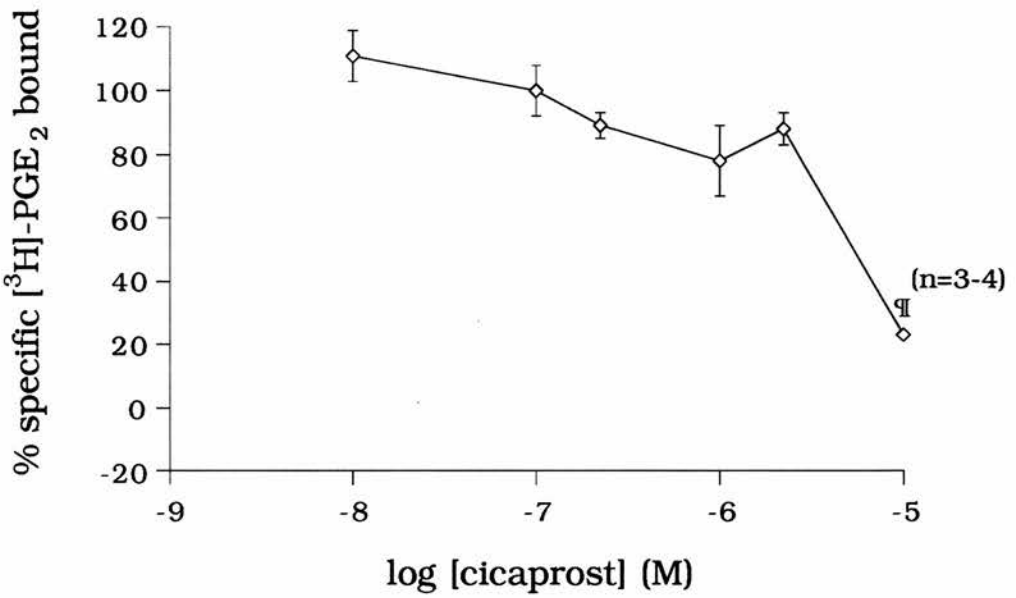


Figure 4.5 Displacement of 2 nM [^{3}H]-PGE_2 from human macrophage-like plasma membranes by the IP - receptor agonist, cicaprost.
¶ ($p<0.01$) denotes significant displacement of specific [^{3}H]-PGE_2 bound.

4.5 DISCUSSION

Since PGs are fatty acid-like molecules, they can be expected to associate non-specifically with hydrophobic membrane components, regardless of a specific binding capacity. This creates problems with PG binding studies, which may be overcome by the preparation of a purified and concentrated plasma membrane fraction, as detailed in the methods section. Indeed, experiments carried out with crude homogenate produced only half the specific binding of that following the sucrose density gradient centrifugation step. When optimum conditions were achieved, approximately 70 % specific binding was routinely obtained. Insufficient protein prevented the use of fresh human monocytes separated from whole blood by Histopaque, whilst the use of cell culture allowed more control over reproducibility of conditions and protein content.

Analysis of the saturation experiments conducted with 0.1 nM to 100 nM [^{3}H]-PGE_2 has suggested the presence of two receptor binding sites on the purified plasma membrane preparation of the differentiated HL-60 cells (Figure 4.1 (a),(b)), one of high affinity, K_d = 2.5 ± 0.4 nM, and a second much lower affinity site. The value of K_d determined by computer analysis is not significantly different at 7.3 ± 2.6 nM. However, the determination of K_d from displacement studies appears to be at odds with these findings.

Displacement of 2 nM [^{3}H]-PGE_2 by PGE_2 itself, revealed an IC_{50} value of 17 ± 1 nM (n=5), with > 90 % displacement occurring over 3 log units of concentration (Figure 4.2). Given that $K_d = IC_{50}$ - [L] when [^{3}H]-L and L have the same physical and chemical properties (Cheng & Prusoff, 1973), K_d = 15 ± 1 nM. This value of K_d is significantly different ($p<0.01$) from that determined from the saturation studies. Since binding to the low affinity site may influence estimation of the K_d by this method, the data was analysed by computer program. In the logistic fit of the data, if P=-1 there is no point trying to look for two sites, 'P' in the logistic equation corresponding to the Hill coefficient

(Barlow & Blake, 1989). In the self displacement of PGE_2 there is no evidence of two sites, and K_d is represented by 26 ± 5 nM which is not significantly different from the value determined from the IC_{50}. There must therefore be an alternative explanation for the discrepancy between these results and those obtained from analysis of the saturation data.

Whilst the effect of overestimating free ligand concentration at equilibrium is most pronounced at the lowest ligand concentrations, this is not likely to be of significance here, since less than 10 % of the total ligand added is bound at any one time. Two binding sites may have been revealed if experiments involving displacement of a higher concentration of [^{3}H]-PGE_2 were conducted. Nevertheless there is a 3- to 6- fold lower evaluation of the K_d from the saturation studies performed at Allergan compared to the displacement studies conducted at Edinburgh.

Interestingly, in the saturation binding studies representing n = 7, the specific binding of 2 nM [^{3}H]-PGE_2 represented 77 ± 5 % of the total binding, at 2453 ± 482 DPM and 146 ± 27 fmoles / mg protein. In contrast, displacement studies using a fresh subculture, whilst producing a similar degree of specific binding represented by 69 ± 2 % at 2 nM, were equivalent to only 583 ± 52 DPM and 60 ± 9 fmoles / mg protein. This suggests a different distribution of binding sites on the membrane preparation prepared here, compared to that isolated from the HL-60 cell line at Allergan. It therefore appears that whilst all HL-60 cells originate from one source, different subcultures may differ in their receptor binding sites. It is also interesting to note, the different estimations of protein content according to the method of assay. At Edinburgh, 6 protein samples were assayed by both the Coomassie Blue (Bradford, 1976) method and the Lowry (1951) method, giving 10 ± 2 μg and 19 ± 3 μg respectively. The Bio-Rad technique used at Allergan is based on the Bradford method.

In further binding studies I measured the displacement of 2 nM [^{3}H]-PGE_2 from human macrophage-like plasma membranes by a series of competing ligands, which are PGE analogues of differing

selectivity for the three PGE receptor subtypes so far characterised.

Nat 11-deoxy PGE_1, an EP_2-selective analogue, displaced [^{3}H]-PGE_2 with an IC_{50} = 30 ± 17 nM (n=4) (Figure 4.2). The fact that this potency, K_i = 27 ± 15 nM, was comparable to PGE_2 itself was not surprising, since PGE_2 has been reported to elevate cyclic AMP levels in the macrophage, an effect classically mediated by the EP_2-receptor subtype. The logistic fit with this data did however suggest two receptor binding sites. Since 13 data points were determined with 11-deoxy PGE_1 as compared to 7 with PGE_2 itself, perhaps smaller increments in the concentration of PGE_2 are necessary to demonstrate the presence of a second much lower affinity site in this preparation. In addition the method of filtration for separation of bound from free may not be fast enough to prevent significant dissociation of the ligand from a lower affinity site. Tests may be carried out using the the more rapid method of centrifugation for separation, in order to determine if this is indeed the case.

A contrasting profile was however observed with two other EP_2-selective analogues, butaprost and AH 13205. In the presence of these competing ligands, displacement at a concentration of 10 μM represented that observed for PGE_2 and 11-deoxy PGE_1 at approximately 10 nM (Figure 4.3). Butaprost and AH 13205 were therefore approximately 1000 times less potent than either PGE_2 or 11-deoxy PGE_1 in displacing [^{3}H]-PGE_2 from this plasma membrane fraction.

Gardiner (1986) has claimed butaprost to be a specific EP_2-receptor agonist. Indeed, on the cat trachea, an EP_2-receptor - containing preparation, butaprost and 11-deoxy PGE_1 are of similar potency (Dong *et al.*, 1986). However butaprost is 500 times less potent than *nat* 11-deoxy PGE_1 in relaxing the histamine-contracted rabbit jugular vein, suggesting the presence of a different EP-receptor on this preparation (Lawrence & Jones, 1992). The jugular vein is highly sensitive to the relaxant action of PGE_2 and the equipotent molar ratio (e.m.r.) of nat 11-deoxy PGE_1 relative to PGE_2 on this preparation is 1.4, as

compared to 685 for butaprost. The binding studies may therefore reflect an EP_2-subtype similar to that on the rabbit jugular vein. A measure of the relative potency of AH 13205 on the jugular vein would be of help in further reconciling these results.

Sulprostone, an EP_1- and EP_3-selective agonist, also showed no appreciable displacement of the specifically bound [^{3}H]-PGE_2 until the 10 μM concentration (Figure 4.4). As a measure of its EP_3 potency relative to PGE_2, inhibition of the twitch response of guinea-pig vas deferens to electrical field stimulation reveals an e.m.r. of approximately 0.1 (Lawrence *et al.*, 1992). Were there an EP_3 component involved, displacement of the [^{3}H]-PGE_2 would therefore be expected at 10-fold lower concentrations than that obtained for PGE_2 itself. Clearly this is not the case.

AH 6809 is an EP_1 antagonist with a pA_2 of ~ 7.0 for PGE_2 on the guinea-pig ileum and fundus and the dog fundus (Coleman *et al.*, 1985a). Binding studies carried out with a 10 μM concentration revealed slight displacement of the specifically bound [^{3}H]-PGE_2 (Figure 4.4). The pA_2 value suggests an IC_{50} value of approximately 100 nM, were there an EP_1-receptor subtype present on this preparation. Insufficient displacement prevented an estimation of a value for IC_{50}.

To test the specificity of the [^{3}H]-PGE_2, displacement studies were also carried out with the specific IP-receptor agonist cicaprost (Dong *et al.*, 1986). Its potency in displacing was shown to be approximately 300 times less than that for PGE_2 (Figure 4.5) represented by an IC_{50} of 4.8 ± 1 μM (n=3-4). This indicated that [^{3}H]-PGE_2 was binding to an EP-receptor for which cicaprost had a weak affinity, with a K_i of 4.2 ± 0.6 μM.

Following the suggestion of an additional EP-receptor subtype at which AH 23848B may be an antagonist (Louttit *et al.*, 1992a,b), two representative experiments were therefore carried out in order to determine if AH 23848B would displace the specifically bound [^{3}H]-PGE_2 from the macrophage-like membrane preparation. Since it is a weak antagonist, pA_2 ~ 5.36, relatively high concentrations were required in order to obtain any

significant displacement. At 10 μM AH 23848B, no significant displacement of the [^{3}H]-PGE_2 was obtained, two separate experiments displacing 51 % and 46 % at 30 μM, and 91 % and 83 % at 100 μM. AH23848B was initially developed as a thromboxane antagonist, therefore the displacing ability of the widely used thromboxane antagonist GR 32191 was also tested in the same two preparations. At 100 μM GR 32191 displaced the specifically bound [^{3}H]-PGE_2 by 26 % and 29 %. These preliminary studies indicate weak affinity of these compounds for the EP receptor, with AH 23848B being the more potent displacer. Further studies are required before any sound conclusions may be drawn as regards the presence of an EP_4-receptor subtype on this preparation.

The time-course study suggests the onset of PGE_2 binding is slow ($t_{1/2}$ ~ 20 min). Since

$t_{1/2}$ onset = ln 2 / onset rate constant

and

onset rate constant = $(k_{+1} \times [L]) + k_{-1}$

then

$(k_{+1} \times [L]) + k_{-1} \sim 0.6 \times 10^{-3}\ s^{-1}$ (1)

As $K_d = k_{-1} / k_{+1}$ $k_{-1} = (K_d \times k_{+1})$

$= 2.5 \times 10^{-9} \times k_{+1}$ (2)

Substituting (2) into (1)

$3.5 \times 10^{-9} \times k_{+1} \sim 0.6 \times 10^{-3}\ s^{-1}$

therefore $k_{+1} = 2 \times 10^5\ M^{-1}s^{-1}$

and $k_{-1} = 5 \times 10^{-4}$ s-1

A forward rate constant (k_{+1}) of $2 \times 10^5\ M^{-1}s^{-1}$ is quite low. Many investigators use values of 10^6 - $10^7\ M^{-1}s^{-1}$ (Yamamura *et al.*, 1985). However, other workers have reported relatively slow association of PGE_2 with its receptor. Indeed Grandt *et al.* (1982) showed that the slow association of PGE_2 to

hamster adipocytes was accelerated by guanine nucleotides and monovalent cations, also more recently reported for human myometrial PGE_2 receptors (Thaler-Dao *et al.*, 1992). Other workers have observed a marked effect of temperature on the association rate for binding (see Brunton *et al.*, 1976). Rao (1974) gives a value of 1.9×10^5 $M^{-1}s^{-1}$ for the association rate constant for the interaction of PGE_2 with the bovine corpus luteum cell membrane preparation, and a value of 3×10^5 $M^{-1}s^{-1}$ has been reported for the porcine fundic mucosal homogenate (Beinborn *et al.*, 1988).

Several binding studies have previously been carried out in an attempt to characterise the PGE receptor present on the mature macrophage and its predecessor, the blood monocyte. Opmeer *et al.* (1983a) present direct evidence for the presence of selective binding sites for $[^3H]$-PGE_2 on rat peritoneal macrophages with a K_d of 32 nM. The competing ligands displaced in the manner $PGE_2 > PGE_1 > PGI_2$, the IC_{50} for PGE_2 displacing 5 nM $[^3H]$-PGE_2 being 100 nM. This represents a 6-7 times lower affinity binding site than on the plasma membrane - rich fraction of the differentiated HL-60 cells. PGI_2 had a relatively low affinity represented by an IC_{50} of 55 μM, hence a K_i of 48 μM, and since it competes for $[^3H]$-PGE_2 in a non-parallel manner compared to PGE_2 itself, it is proposed to have a separate binding site. Considering the relationship between K_d and IC_{50} when the $[^3H]$-ligand and competing ligand have the same biological profile, $K_d = IC_{50} - [L]$, the IC_{50} for displacement of $[^3H]$-PGE_2 by PGE_2 itself represents a K_d of 95 nM, which is three-fold greater than that obtained from the saturation experiments. A better correlation would result if all the points are taken into consideration in the displacement study (Opmeer *et al.*, 1983a, Figure 3), and would also reduce the degree of non-parallelism between the displacement of $[^3H]$-PGE_2 by PGE_2 and PGI_2.

A complementary study using 20 nM $[^3H]$-PGI_2 as the ligand, was subsequently carried out by the same group (Opmeer *et al.*, 1983b). PGE_2 and PGI_2 displaced the specifically bound $[^3H]$-PGI_2 in both

the whole cell and membrane-enriched fractions, PGE_2 being the more potent of the two. This order of potency was also observed for prostaglandin activation of cyclic AMP in the macrophages, which takes into account the affinity and efficacy of the compounds. The greater affinity of PGE_2 and its stronger ability to elevate cyclic AMP when compared to PGI_2, suggests the [^{3}H]-PGI_2 to be binding to an EP receptor. It is however difficult to be confident about any conclusions since there is no indication of the error involved in either of the binding studies. The IC_{50} values obtained for PGE_2 on whole cells and membrane fractions were 2.5 nM and 2 nM respectively. In contrast, PGI_2 displaced with IC_{50} values of 6 nM and 400 nM, the displacement from whole cells being biphasic. Given as before that $K_d = IC_{50} - [L]$ when the competing ligand is of the same biological activity to the [^{3}H] - ligand, it is difficult to determine a K_d value for PGI_2 on the whole cells when an IC_{50} of 6 nM is obtained in displacing 20 nM [^{3}H]-PGI_2. The seventy-fold loss of potency on the membrane fraction is thought to reflect a loss of PGI_2 activity through degradation or inactivation. These results may suggest the existence of PGE_2 receptors on rat peritoneal macrophages for which PGI_2 has a lower affinity, rather than separate receptors for PGE_2 and PGI_2.

The PGE_2 receptor on human peripheral blood monocytes has also been characterised (Eriksen *et al.*, 1985). Steady state binding of [^{3}H]-PGE_2 was obtained by 30 min at 15 °C, 22 °C and 37 °C. The observation that $PGF_{2\alpha}$ and arachidonic acid were more than 4-fold less potent than PGE_2 in displacing 0.6 nM [^{3}H]-PGE_2, and did not stimulate cyclic AMP levels, suggested the binding of [^{3}H]-PGE_2 to be specific. Further analysis of this binding data and its transformation to a Scatchard plot revealed a single site with a value for K_d of 1.1 nM, and B_{max} of 4.1 fmol / 10^7 cells. The concentration of PGE_2 producing 50 % of the maximum adenylate cyclase response was ~ 6 nM, suggesting a relationship between the PGE_2 receptor and adenylate cyclase in the cell membrane. The PGE receptor demonstrated on these monocytes is shown to be of greater affinity than that isolated from differentiated HL-60

cells. This difference may reflect changes which occur during the course of maturation, or a whole cell preparation as compared to a plasma membrane fraction.

In studies involving a murine macrophage-like cell line, P388 D_1, binding to intact cells revealed two PGE_2-receptor binding sites (Fernandez-Botran & Suzuki, 1984a). The high-affinity receptor had a K_d of 1.1 nM and B_{max} of 3.9 fmol / 10^8 cells, and the low-affinity receptor a K_d of 20 nM and B_{max} of 24 fmol / 10^8 cells. Competitive binding studies indicated PGI_2 and $PGF_{2\alpha}$ were respectively 10 times and 100 times less effective than PGE_2 in displacing 1 nM [^{3}H]-PGE_2 thereby confirming the presence of an EP-receptor. The K_d for the high affinity binding site correlates well with that on the human monocyte, though the density of receptors is much greater on this cell line. The low affinity receptor is comparable to that identified on the rat peritoneal macrophage. Partially purified membrane fractions of P388D_1 cells obtained by sucrose density gradient centrifugation, have been used for the determination of adenylate cyclase activity (Fernandez-Botran & Suzuki, 1984b). It is apparent from these studies that the low-affinity receptor is probably the more important for transducing the signal, since the biologic effects of exogenously added PGE_2 are at 0.1 μM to 1 μM.

In a more recent study of the PGE_2 binding sites on macrophage-like P388D_1 cells, IL-1 is shown to reduce the binding capacity of both the high, 0.31 to 0.12 fmol / 10^6 cells, and low affinity sites, 2.41 to 1.51 fmol / 10^6 cells, in a concentration-dependent manner without altering the K_d values of either, 2.7 nM and 40 nM respectively (Rae *et al.*, 1992). The desensitisation of PGE_2 receptors by IL-1 on P388D_1 cells occurs within the range of K_d values, suggesting a receptor-ligand interaction. PGE_2 is reported to induce desensitisation in P388D_1 cells in two steps, the early homologous desensitisation whereby only subsequent effects of PGE_2 are impaired, and the later heterologous desensitisation influencing the effects of more than PGE_2 alone (Shamma *et al.*, 1988). It is proposed that this may represent a mechanism by which IL-1 can overcome the

immunosuppressive actions of PGE_2, and reflects the complex nature of the control processes functioning in the immune system.

Earlier studies demonstrated pharmacological amounts of PGE_1 to suppress acute and chronic inflammation *in vivo* in adrenalectomised rats (Zurier *et al.*, 1973), and the phosphodiesterase inhibitor theophylline to enhance the inhibitory effect of PGE_1 in adjuvant-induced arthritis in rats (Bonta *et al.*, 1978). Indeed, PGs of the E series are known to exert their anti-inflammatory effects through stimulation of cyclic AMP levels *in vitro* (Pelus & Strausser, 1977). Secretion of IL-1 from macrophages stimulates the release of PGE_2 from many cell types, including other macrophages, the resulting elevation of cyclic AMP due to the PG stimulation of receptor-coupled adenylate cyclase of macrophages being associated with the inhibition of several functions of these phagocytes (Bonta & Parnham, 1982; Kammer, 1988).

PGE_2 has been shown to have a biphasic effect on the adenylate cyclase system of rat peritoneal macrophages, inhibiting PGI_2-induced elevation of cyclic AMP at low concentrations, and increasing cyclic AMP levels at higher concentrations (Adolfs & Bonta, 1982). In the presence of 1.4 nM to 12 nM PGE_2, a concentration range which is itself unable to significantly raise cyclic AMP levels, the stimulatory response to 2.8 μM PGI_2 and a stable synthetic analogue, DDH-carbo-PGI_2, was inhibited in a dose-dependent manner. Concentrations greater than this for PGE_2 caused a dose-dependent increase in cyclic AMP levels. At 56 nM PGE_2 the inhibitory influence on DDH-carbo-PGI_2 was not evident, either not existing or being masked by the PGE_2-induced stimulation of cyclic AMP. Perhaps the initial inhibitory and later stimulatory influence of PGE_2 on adenylate cyclase reflect subtypes of the PGE receptor, positively and negatively coupled to adenylate cyclase. At the lower concentration range, PGE_2 may occupy those negatively coupled, thereby inhibiting the PGI_2- or DDH-carbo-PGI_2- induced elevation of cyclic AMP. A threshold concentration may be reached when EP receptors that are positively coupled to adenylate cyclase, and for which PGI_2 has a

low affinity, are occupied. A more comprehensive study of the PGE receptors present on the rat peritoneal macrophages is therefore necessary for a greater understanding of the activity of PGE_2.

A comparison has been made between human and rat macrophages with regard to the regulation of cyclic AMP by the prostaglandins (Bonta *et al.*, 1984). Peritoneal macrophages were isolated from the rat, and from dialysates of patients undergoing continuous ambulatory peritoneal dialysis (CAPD). In contrast to rat peritoneal macrophages, those isolated from humans are reported to have separate binding sites for PGE_2 and PGI_2. The rat macrophages were more sensitive to PGE_2 than to DDH-carbo-PGI_2 which correlates with its greater affinity relative to PGI_2 in the binding studies already described. In contrast, whilst the potency of the response to DDH-carbo-PGI_2 was similar to that on rat cells as measured by the elevation of cyclic AMP levels, there was a much smaller effect in the presence of PGE_2 with respect to the human macrophages. PGE_2 has been proposed as the major PG in exerting effects of importance in governing the cyclic AMP-mediated functions of macrophages (Bonta & Parnham, 1982; Gemsa *et al.*, 1982). These studies on human macrophages suggest an important role for PGI_2, though it is not known what bearing the origin of these macrophages has on the receptor adenylate cyclase system. However, cells from sites of infectious inflammation show a greater increase in the sensitivity of adenylate cyclase to PGE_2 than to PGI_2 (Adolfs *et al.*, 1985). In this study, human peritoneal macrophages were again isolated from CAPD patients, though besides the macrophages isolated in the absence of complications, as the first study described, macrophages were also isolated from these patients during inflammation and after recovery. Whilst macrophages from the uncomplicated stage of CAPD were more responsive to DC-PGI_2 than PGE_2 as already reported, during inflammation there was a marked increase in the sensitivity to PG stimulation, especially with PGE_2. Perhaps then PGE_2 is predominantly more important in conditions of inflammation.

Indeed, modulation of granulomatous inflammation by PGE_2

depends upon the period of exposure to the PG (Parnham *et al.*, 1979). A single daily dose of PGE_2 for the first three days enhanced the granuloma formation as measured on day 8, and inhibited the formation if administered on days 4-7. Consequently, treatment with PGE_2 during the whole time period showed no significant change in the dry granuloma weight indicating that the later inhibitory action of PGE_2 had reversed its early stimulatory effect. PGI_2, 6-keto $PGF_{1\alpha}$ and $PGF_{2\alpha}$ had no significant effect on the granuloma formation when administered over the 4-7 day period. More recently Bonta *et al.* (1981) have related the effects of PGE_2 on the adenylate cyclase system *in vitro* to chronic inflammation *in vivo*. They suggest the time-dependent increase in *ex-vivo* responsiveness of granuloma-derived macrophages to PGE parallels the anti-inflammatory effect of PGE on the granuloma tissue. A reduction in the level of cyclic AMP during the inflammatory period was accompanied by a fall in the endogenous synthesis of prostaglandins. In particular the effect of PGE_2 on the cyclic AMP levels was considerably more pronounced than with macrophages isolated before this period.

The mechanism of activation of adenylate cyclase is likely to be explained by the ternary complex model. The components of this include the receptor, adenylate cyclase and the guanine nucleotide regulatory protein, or G protein, which transmits the signal from the receptor to the adenylate cyclase. Indeed PGE_1 requires guanine nucleotides to stimulate the adenylate cyclase in guinea-pig peritoneal macrophage membranes, and enhances the GTP- and Gpp(NH)p -, a non-hydrolysable analogue of GTP, stimulated cyclic AMP levels (Verghese & Snyderman, 1983). In the presence of GTP, the G protein dissociates into G_α and $G_{\beta\gamma}$, as discussed in chapter 3 of this thesis. At the same time the PGE_2 binding site has been reported to be converted to both a lower (adrenal medulla, kidney medulla, brain, hypothalamus) or higher (adipocytes, myometrium) affinity form. Lerner *et al.* (1992) have shown a nonhydrolysable form of GTP to shift $[^3H]PGE_2$ binding from two sites, one of high affinity and the other of low affinity, to one site of intermediate affinity. Binding studies were therefore

carried out in the presence and absence of a non-hydrolysable analogue of GTP to determine if the receptor was indeed G protein linked. A preliminary study revealed no change in the IC_{50} value for displacement of 2 nM [^{3}H]-PGE_2 by PGE_2 itself, in the presence of 100 μM GTP-γ-s.

Additional displacement studies and assessment of the activity of these PGE analogues on the pig saphenous vein preparation are required for a more comprehensive EP-receptor characterisation. This should involve the use of other analogues such as the EP_2- and EP_3- selective analogue misoprostol, especially given the recent evidence for its immunomodulatory role (Widomski *et al.*, 1991), and compounds active at DP-, FP- and IP-receptors to further test the selectivity of binding.

Binding studies primarily produce estimates of affinity, the intrinsic efficacy parameter being essentially inaccessible. The advantage of binding over an isolated preparation is the ability to estimate receptor density, however because of the coupling system between receptor occupation and tissue response one cannot assume a direct correspondence between the proportions of heterologous receptor populations and the pharmacological response. Such binding studies are often accompanied by second messenger and / or functional studies to confirm the presence of a receptor. Preliminary experiments for determination of cyclic AMP levels in whole cells were carried out using the automated Attflo system at Allergan Pharmaceuticals. Basal levels of cyclic AMP in undifferentiated HL-60 cells are not elevated by PGD_2 or $PGF_{2\alpha}$ indicating a degree of specificity. PGE_2 and 11-deoxy PGE_1 at 100 μM increased levels to approximately 105 pmoles / mg protein and 50 pmoles / mg protein respectively. The ED_{50} value, representing the concentration at which a half-maximal cyclic AMP level is attained, was 1 μM in each case. In differentiated cells the maximal response to PGE_2 was reached at 1 μM and represented an increase of 20 pmoles cyclic AMP / mg protein above basal (14 ± 5 pmoles / mg). This represents only a third of the response in the undifferentiated form, and has an ED_{50} of ~ 200 nM.

Sucrose density gradient centrifugation has previously been used to isolate plasma membranes from various cell types. In the case of mouse lymphoid cells (Koizumi *et al.*, 1981), electron microscopy and measurement of the specific activities of the marker enzymes for plasma membrane, (Na^+ / K^+)-ATPase, Mg^{2+}-ATPase and 5'-nucleotidase, indicated the plasma membrane - rich fraction had been isolated at the interface between 20 % and 40 % sucrose in the gradients. Whilst isolating the plasma membrane - rich fraction from the murine macrophage-like cell line $P388D_1$ between 30 % and 45 % sucrose (Fernandez-Botran & Suzuki, 1984a), the results of marker enzyme activity suggested this to be only a partially purified preparation. The interface used here between 0.9 M sucrose and 1.2 M sucrose corresponds to that between 30 % and 40 % respectively, and therefore falls within that already defined for plasma membrane isolation. A further study of the adenylate cyclase activity of the membrane fraction should confirm that it is indeed membrane-rich, since adenylate cyclase is a membrane bound enzyme. In addition, 5'-nucleotidase, 5'-ribonucleotide phosphorylase, is a plasma membrane-associated enzyme that because of its high activity in monocytes or macrophages can be used as a marker for these cell types (Breitman, 1990). The assay involves a measure of the capacity to release inorganic phosphate from adenosine monophosphate.

The results discussed here suggest that a further subtype of the EP receptor may exist. Additional binding and second messenger studies are required in order to reconcile these results with those found on the rabbit jugular vein and the more recently communicated pig saphenous vein. There appear to be problems in using different subcultures of a cell line. It is possible that the discrepancies may be more pronounced in studies dependent on such processes as induced differentiation. Using one source and restricting experiments to a range of passages in which the cells are known to exhibit 'typical' characteristics may help to overcome such irregularities.

5 Summary

The advent of the NSAIDs and the premise that their mechanism of action was through inhibition of cyclooxygenase activity (Vane, 1971), suggested that the prostanoids were pro-inflammatory. Indeed prior to this PGE_2 was identified in rat (Willis, 1969a,b) and human (Greaves & Søndergaard, 1970; Greaves *et al.*, 1971) inflammatory exudate, and was also shown to induce signs of inflammation in both (Crunkhorn & Willis, 1969,1971a). Subsequently PGE_2 has been implicated as a mediator in several models of inflammation, as discussed in chapter 2 of this thesis. Besides this pro-inflammatory activity of PGE_2, its pro-aggregatory effect on platelets is of great interest (chapter 3) since inflammatory mediators are released following platelet activation.

Nonetheless, there have been indications that all the beneficial effects of the NSAIDs are not mediated by inhibition of prostanoid biosynthesis, such as the inhibition of neutrophil activation (Rampart & Williams, 1986a; Weissman, 1987; Abramson & Weissmann, 1989). In addition, as early as 1976 there was a report on the ability of PGE_1 and PGE_2 to suppress lymphocyte activation (Bray *et al.*, 1976), prompting the first suggestion that the PGEs may be more accurately described as modulators of inflammation rather than mediators. Further evidence for this anti-inflammatory effect of PGE_2 is detailed in chapter 4.

Distinct receptors are known to exist for each of the five naturally occurring prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2. In addition the PGE receptor has been further classified into at least three subtypes (Bennet & Posner, 1971; Coleman *et al.*, 1980; Dong *et al.*, 1986; Coleman *et al.*, 1987a,b,c) believed to be coupled to separate second messenger systems (Gutman *et al.*, 1979; Creese & Denborough, 1981). In the absence of specific agonists or antagonists, the synthesis of PGE analogues showing differing selectivity for the EP_1-, EP_2- and EP_3- receptor subtypes has

proved a useful tool in characterising the subtype(s) involved in a particular system.

It has been suggested that the pro-inflammatory activity of PGE_2 is the result of its vasodilatory activity. However, we have compared potentiation of the exudation response to bradykinin and FMLP in a rabbit model of skin inflammation by PGE_2, a range of PGE analogues and cicaprost, a specific IP-receptor agonist, and related this to their ability to increase local blood flow. These studies suggest that whilst vasodilatation may have an important role in the PGE_2-induced potentiation, another mechanism may also be involved.

Vasodilatation is associated with a rise in the second messenger cyclic AMP, formed as a result of adenylate cyclase activity believed to be coupled to the EP_2-receptor subtype. Indeed, this may be responsible for the potentiation observed above the 10 ng dose of PGE_2. However, an EP_3-mediated effect possibly involving chemotaxis of neutrophils may account for the potentiation at lower doses, in the absence of any significant increase in local blood flow.

The ability to induce vasodilatation does not necessarily result in potentiation of plasma exudation, since β-adrenoceptor agonists, whilst causing vasodilatation, inhibit increased vascular permeability (Whelan & Johnson, 1992). This is believed to be a direct effect on the endothelial cells, and may also be a possible mechanism for anti-inflammatory effects of PGE_2. In addition, perhaps PGE_2 potentiates plasma exudation in this rabbit skin inflammation model in part through a direct action on the microvascular membrane, as suggested for PGE_1 in the canine forelimb (Amelang *et al.*, 1981) and previously demonstrated by fluorescence studies with PGE_1, PGE_2 and $PGF_{2\alpha}$ in the hamster cheek pouch (Svensjo, 1978).

The pro-aggregatory effect of PGE_2, suggested to result from an inhibition of adenylate cyclase activity, may also be mediated by the EP_3-receptor subtype. This is supported both by potentiation of small reversible waves to PAF and inhibition of cicaprost-induced increases in cyclic AMP. Whilst there is no evidence for the

presence of an EP_2-receptor subtype mediating an anti-aggregatory effect through an increase in cyclic AMP, PGE_2 and the PGE analogues may exert such an effect through interaction with the IP-receptor at the higher concentrations. This caused some problems with the aggregation studies, which were overcome in the second messenger studies. The reasoning was that any small increases in cyclic AMP induced by PGE_2 or the PGE analogues would be masked by the much greater increase induced by cicaprost.

Whilst a pro-inflammatory effect of PGE_2 is observed when administered i.d. into rabbit skin, other models where PGEs were administered systemically demonstrated anti-inflammatory responses, possibly through inhibition of mediator release. In particular, PGE_2 is reported to inhibit IL-1 release from macrophages and subsequently the IL-1 - induced IL-2 production. This anti-inflammatory effect of PGE_2 is believed to be mediated by an EP-receptor subtype other than the EP_1-, EP_2- and EP_3-receptor subtypes so far classified. Binding studies were carried out on a purified plasma membrane preparation from HL-60 cells differentiated into human macrophage-like cells.

Only PGE_2 and 11-deoxy PGE_1, an EP_2-selective analogue, inhibited the specific binding of [^{3}H]-PGE_2 out of a range of PGE analogues tested, including others also selective for the EP_2-receptor subtype. Preliminary studies using a PGE analogue reported to be a weak antagonist at the putative EP_4-receptor subtype (Louttit *et al.*, 1992a,b), suggest that this additional EP-receptor subtype may indeed be involved in this anti-inflammatory effect of PGE_2. Further studies testing the full range of PGE analogues available on the pig saphenous vein preparation containing this novel receptor subtype would obviously be of great benefit.

The presence of PGE-receptor subtypes can account for some of the often opposing effects of PGE_2. Characterisation of these subtypes is important for the development of therapeutically useful PGE analogues. There appears to be an ever expanding range of PGE analogues selective for the three receptor subtypes so far

characterised, and their selectivity for the putative EP_4-receptor subtype will no doubt only be a matter of time. Compounds of greater specificity would be of tremendous benefit in prostanoid research, especially antagonists specific for each of the subtypes characterised.

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CHARACTERISATION OF PGE_2 RECEPTORS MEDIATING INCREASED VASCULAR PERMEABILITY IN INFLAMMATION

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PGE_2 has little effect on vascular permeability when injected alone into rabbit skin, but potentiates the local exudation produced by other inflammatory mediators. This has been attributed to its vasodilator activity (5). However, there are potential difficulties with using PGE_2 as an agonist since it is known to act on (at least) three receptor subtypes, designated EP_1, EP_2 and EP_3 (3,4). We have investigated the ability of more selective PGE analogues to potentiate bradykinin (BK)-induced plasma exudation in rabbit skin (leakage of ^{125}I albumin (6)).

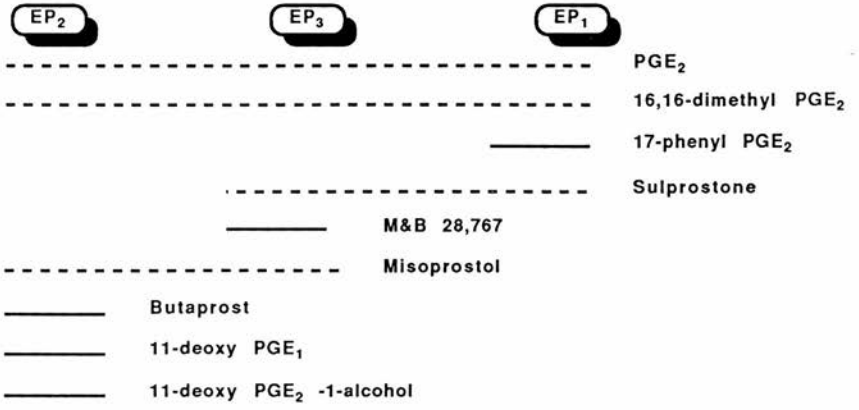

FIG. 1 Specificity of action of the PGE analogues used in this study.

EP_1 receptors mediate contraction of the guinea-pig ileum, fundus and trachea *in vitro*; the action of PGE_2 is antagonised by SC 19220 and AH 6809 (2). EP_2 receptors mediate relaxation of cat trachea, guinea-pig trachea and rabbit jugular vein *in vitro*, and dog hind limb

arterial vessels *in vivo*;. EP_3 actions are characterised *in vitro* by contraction of the chick ileum, inhibition of transmitter release in the guinea-pig vas deferens and inhibition of secretion in the rat isolated gastric mucosa. No antagonists are available for EP_2 or EP_3 receptors.

PLASMA EXUDATION BY EP_2 AGONISTS

We set up the rabbit skin model in the expectation that we would readily characterise the prostanoid receptor mediating the potentiation of exudation as an EP_2 receptor, since vasodilatation is the archetypal EP_2 effect. However, the initial results were surprising in that the potent EP_2 agonists butaprost (1000 ng), 11-deoxy PGE_1 (5000 ng) and 11-deoxy PGE_2-1-alcohol (5000 ng) were poor potentiating agents, achieving +69%, +98% and +38% of the BK control respectively. Only misoprostol (1000 ng) could achieve the maximal potentiation seen with PGE_2 (+265% at 1000 ng) (FIG. 2). Furthermore, whereas PGE_2 at only 1 ng gave a potentiation of +108%, the EP_2 agonists showed little activity below concentrations of 100-1000 ng. The weak effects were not due to a partial agonist action, since pre-treatment with 11-deoxy PGE_2-1-alcohol (5000 ng intradermally) did not antagonise potentiation by PGE_2 or misoprostol, but produced additive effects (n=4).

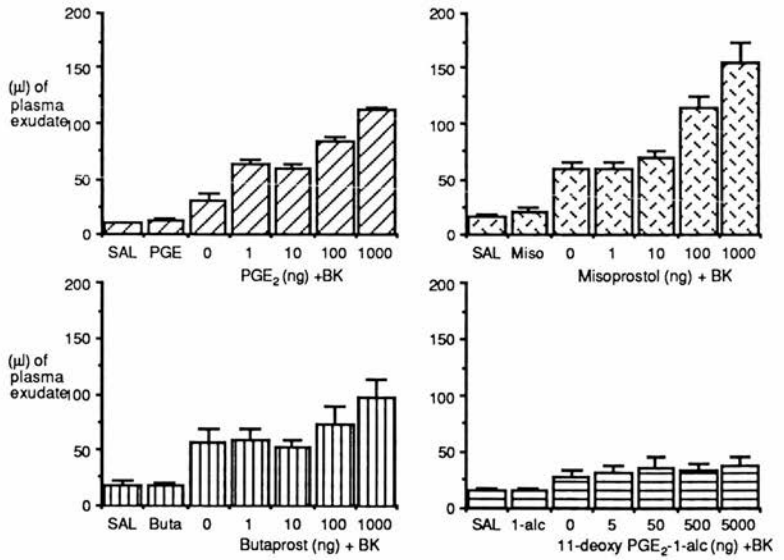

FIG 2. The ability of PGE_2, misoprostol, butaprost and 11-deoxy PGE_2-1-alcohol to potentiate plasma exudation in rabbit skin induced by intradermal bradykinin (500ng). SAL and Drug columns represent injections of saline and drug at the highest concentration tested, but in the absence of BK. Values are the mean ± s.e.m of at least 4 experiments.

PLASMA EXUDATION BY EP$_3$ AGONISTS

EP$_3$ activity was examined using sulprostone and M&B 28,767, whose true activity could be assessed only after its thromboxane (TP) agonist activity had been blocked by the TP receptor antagonist GR 32191 (2 mg/kg i.v.). M&B 28,767 (1 ng) significantly increased plasma exudation to +78% (FIG. 3) (as compared to +20% in the absence of GR 32191). Unfortunately TP receptor block is difficult to achieve in the rabbit, and a dose-ratio of only 15 was achieved against U 46619-induced pulmonary hypertension. While higher concentrations of M&B 28,767 may overcome the TP block, neither M&B 28,767 nor sulprostone showed further potentiation with higher concentrations (100-1000 ng) and both are devoid of EP$_2$ activity.

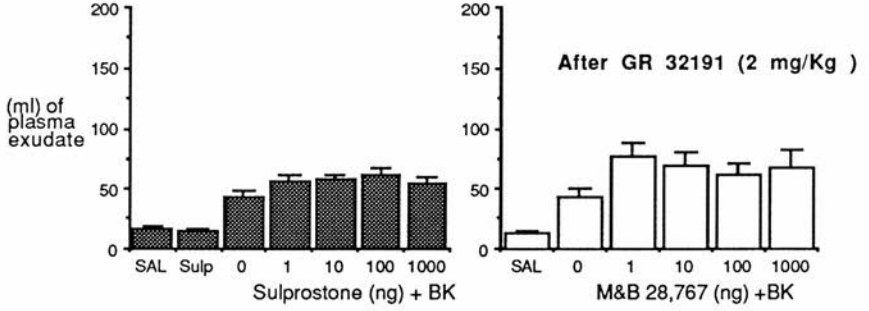

FIG. 3. The ability of sulprostone and M&B 28,767 (after GR 32191) to potentiate plasma exudation. Conditions as in FIG. 2.

DILATATION IN THE SKIN

FIG. 4 shows dilatation in the skin, measured by a ^{133}Xe clearance technique (6).

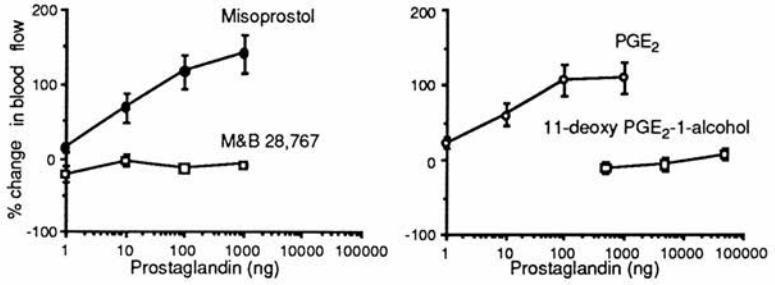

FIG. 4. The ability of PGE$_2$, misoprostol, M&B 28,767 (in the presence of 2mg/kg GR 32191) and 11-deoxy PGE$_2$-1-alcohol to increase blood flow in rabbit skin. Drugs were mixed with ^{133}Xe (5-10 μCi per injection) and injected intradermally. Values are the mean ± s.e.m. of at least 4 experiments.

PGE_2 and misoprostol are potent dilators, although at 1 ng PGE_2 produces little dilatation. M&B 28,767(even after TP receptor block) and sulprostone (1000 ng, n=4) did not dilate. The EP_2 agonist 11-deoxy PGE_2-1-alcohol which did not potentiate BK-exudation did not induce dilatation, and butaprost which was slightly more effective, induced some dilatation (50% at 1000 ng,n=3)

CONCLUSIONS

Potentiation of BK-induced exudation by PGE_2 may have two components. Neither is mediated by an EP_1 receptor as 17-phenyl PGE_2 does not potentiate BK in concentrations up to 1000 ng (n=4). The high potency (1-10 ng) component may be mediated by an EP_3 receptor and appears to be unrelated to dilatation. One possibility is that dilatation induced by BK is potentiating an EP_3 receptor-mediated, leukocyte-dependant mechanism, as has been shown with LTB_4(1). The lower potency (100-1000 ng) component may be mediated by an EP_2 receptor and does correlate with dilatation. However, the low potency of butaprost and the inactivity of 11-deoxy PGE_2-1-alcohol suggests that the receptor differs from the conventional EP_2 subtype.

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Potentiation of aggregation and inhibition of adenylate cyclase in human platelets by prostaglandin E analogues

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1 The 16-phenoxy prostaglandin E analogue sulprostone consistently potentiates primary aggregation waves induced by adenosine 5'-diphosphate (ADP), PAF and 11,9-epoxymethano PGH_2 (U-46619) in platelet-rich plasma from human donors. The effect is not blocked by the TP-receptor antagonists, EP 092 and GR 32191. The high potency of sulprostone (threshold concentration = 4–10 nM) and the weak block of sulprostone potentiation by the EP_1-receptor antagonist, AH 6809 ($pA_2 = 4.3$) suggest the involvement of EP_3-receptors as opposed to EP_1- or EP_2-subtypes.

2 Eight prostaglandin E (PGE) analogues were compared against sulprostone for their effects on PAF-induced aggregation in human platelet-rich plasma (PRP) in the presence of GR 32191 and the DP-receptor antagonist, BW A868C. PGE_2 and 11-deoxy PGE_2-1-alcohol showed evidence of both potentiating and inhibitory actions and butaprost showed only inhibitory activity at high concentrations. The remaining analogues always elicited potentiation, with the following potency ranking: sulprostone = 16,16-dimethyl PGE_2 > MB 28767 > misoprostol > GR 63779X = 17-phenyl-ω-trinor PGE_2. The results again indicate that EP_3- rather than EP_1- or EP_2-receptors are involved. However, relative potentiating potency could be affected by differences in plasma protein binding and the very high sensitivity of the human platelet to prostacyclin (IP)-receptor-mediated inhibition (IC_{50} for the specific IP-receptor agonist cicaprost = 0.8 nM).

3 On human washed platelet suspensions the PGE analogues, with the exception of butaprost, inhibited the rise in adenosine 3':5'-cyclic monophosphate (cyclic AMP) induced by cicaprost (8 nM). PGE_2 produced a monophasic inhibition curve (IC_{50} = 5.4 nM, 92% inhibition at 600 nM). The potency ranking was 16,16-dimethyl PGE_2 > sulprostone > MB 28767 = PGE_2 > misoprostol > GR 63778X > 17-phenyl-ω-trinor PGE_2 > 11-deoxy PGE_2-1-alcohol. AH 6809 inhibited the effect of sulprostone and 17-phenyl-ω-trinor PGE_2 with pA_2 values of 5.75 and 5.32 respectively; these values are at least one log unit lower than those found for EP_1-receptor block in smooth muscle.

4 There is a statistically significant correlation between IC_{50} values for the PGE analogues on the human platelet cyclic AMP assay and the guinea-pig vas deferens (standard EP_3 preparation): slope = 1.00, $r = 0.80$, $P < 0.05$. However the correlation is far from ideal and GR 63779X in particular has a lower potency in the cyclic AMP assay. At this time we suggest that it is prudent to describe the human platelet receptor as 'EP_3-like'.

5 We believe that our results provide further evidence for linking PGE-induced potentiation of aggregation to inhibition of adenylate cyclase. Sulprostone is a suitable agonist for further study of this system and in particular the nature of the G-protein linkage(s) involved. In addition the necessity to consider potentiation of platelet aggregation in relation to the clinical use of PGE analogues in man is emphasised.

Keywords: Prostaglandin E_2; prostacyclin; sulprostone; cicaprost; potentiation of platelet aggregation; inhibition of adenylate cyclase; EP-receptors

Introduction

In the early phase of prostanoid research, prostaglandin E_1 (PGE_1) was shown to inhibit platelet aggregation in platelet-rich plasma (PRP) from man, pig and rat (Kloeze, 1967). This effect was associated with a rise in the adenosine 3':5'-cyclic monophosphate (cyclic AMP) level in the platelet (Vigdahl *et al.*, 1969; Robison *et al.*, 1969). Following the discovery of prostacyclin (PGI_2) (Moncada *et al.*, 1976), it became clear that PGE_1 probably behaves as an agonist at prostacyclin (IP-) receptors on platelets (Whittle *et al.*, 1978). This view was strengthened by the demonstration that PGE_1 competes with either [^{3}H]-PGI_2 (Siegl *et al.*, 1979; Eggerman *et al.*, 1986) or [^{3}H]-iloprost (Schillinger & Losert, 1980) for a saturable binding site on the human platelet. PGE_1 is about an order of magnitude less potent than PGI_2 in both functional and binding assays.

The effect of PGE_2 on platelet function is more variable and more difficult to analyse. In PRP from pig and rat, PGE_2 potentiated adenosine diphosphate (ADP)-induced aggregation with threshold activity present at 100 and 10 nM respectively (Kloeze, 1967). In the same studies PGE_2 at 5–10 μM inhibited aggregation in human PRP and Robison and co-workers (1969) showed that cyclic AMP levels were raised at these high concentrations. Inhibition of primary aggregation and enhancement of secondary aggregation to ADP in human PRP have been reported for PGE_2 (Shio & Ramwell, 1972; McDonald & Stewart, 1974), while enhancement of primary phase aggregation has also been observed (Andersen *et al.*, 1980). More recently Gresele and co-workers (1988) showed that PGE_2 (0.5–5 μM) potentiated arachidonate-induced aggregation in human PRP from all 'non-responders to thromboxane synthetase inhibitors', whereas aggregation was inhibited in 80% of the 'responder' group.

It has been suggested that PGE_2 interacts with a specific receptor on the platelet surface to enhance aggregation and its variable effects are due to an opposing (albeit weak) anti-aggregatory action operating through the IP-receptor (Anderson *et al.*, 1980; Tynan *et al.*, 1984). The same

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research group (Eggerman *et al.*, 1986) went on to provide evidence of a specific binding site for [^{3}H]-PGE_2 on human platelets; the radioligand was displaced equally well by PGE_1 and PGE_2 (IC_{50}~10 nM) whereas PGI_2 was relatively ineffective (IC_{50}~3 μM). Gray & Heptinstall (1985) suggested that activation of this receptor leads to inhibition of adenylate cyclase. The studies of Ashby (1988) support this view and point to the regulatory G-protein G_i as the link between receptor and cyclase molecules.

It has been known for some time that certain PGE analogues (e.g. 16-*p*-fluorophenoxy-ω-tetranor PGE_2) can induce irreversible aggregation of human platelets (Jones *et al.*, 1979; MacIntyre *et al.*, 1978) and that this can be attributed (at least in part) to their agonist action at thromboxane (TP-) receptors (Jones *et al.*, 1982). During testing of prostanoids for potential TP agonist activity in human PRP, we observed that another 16-phenoxy PGE analogue, sulprostone, occasionally produced a slow aggregation wave which was not preceded by a shape change signal; the response was not typical of a TP-receptor agonist (e.g. 11,9-epoxymethano PGH_2 (U-46619)), but was similar to that produced by adrenaline in some PRP samples (see Hourani & Cusack, 1991). On further investigation we found that sulprostone at low concentrations potentiated primary aggregation responses to ADP, PAF and U-46619 in PRP from all donors examined. This led us to suggest that sulprostone might be a specific agonist for the 'pro-aggregation receptor' negatively linked to adenylate cyclase. This paper describes experiments designed to test this hypothesis and to characterize the receptor involved.

Methods

Platelet aggregation

The preparation of PRP and washed platelet suspensions (using PGI_2) from human blood and the measurement of shape change and aggregation responses were carried out as recently described by us (Tymkewycz *et al.*, 1991). GR 32191 (500 nM) and BW A868C (200 nM) were added to the bulk of the PRP at the start of the aggregation measurements. For the study of potentiation, the PGE analogue was added to an aliquot of PRP, 2 min before a fixed dose of the aggregating agent and aggregation was measured for a further 1–2 min. The concentrations of ADP (500–800 nM) and PAF (15–50 nM) were chosen to give an aggregation response between 15–20% of the maximum aggregation response for each agent (obtained with 10 and 1 μM respectively). Sulprostone was used as the standard potentiating agonist and a concentration-response curve involving duplicate measurements was obtained for each PGE analogue over a time period when a submaximal potentiation response to sulprostone remained constant. Responses were calculated as a percentage of the control response and an EC_{150} value, representing a 50% increase in aggregation response over control was calculated. Measurements were completed within 2 h of aspiration of the PRP. For the study of inhibition of aggregation in PRP by cicaprost, PGE_1 and PGE_2, ADP at a concentration of 2 μM (in a few instances 4 μM) was used to produce a 75% maximal aggregation response.

Cyclic AMP measurements

Fresh human PRP or time-expired (5 days at room temperature) human platelet concentrates were treated with 10 μM indomethacin and then centrifuged at 450 *g* for 20 min. The pellet was suspended in sufficient 0.05 M Tris buffer pH 7.4 containing 4 mM EDTA at 37°C to give an absorbance at 600 nm of about 1.4 (10 mm pathlength). In 10 experiments using 180 ml of fresh blood, the protein content of the suspension (45 ml), as measured by the method of Bradford (1976), was found to be 1.5 ± 0.1 mg ml^{-1} (mean ± s.e.mean). GR 32191 (0.5 μM) was added to the platelet suspension. Duplicate 0.5 ml aliquots of the suspension were incubated with the varying concentrations of PGE analogue for 2 min, followed by 8 nM cicaprost for 1 min. In each experiment a single concentration of sulprostone (6.45 nM) was included to ensure that the cyclase inhibitory effect was functioning. After quenching with 1 ml ethanol, the precipitated material was sedimented by centrifugation at about 1000 *g* for 30 min. The supernatant was removed, evaporated to dryness and the residue dissolved in 0.25 ml assay buffer. Insoluble material was sedimented by centrifugation at 2300 *g* for 30 min at 4°C. The cyclic AMP content of duplicate 50 μl aliquots of supernatant was assayed by competition with [8-^{3}H]-cyclic AMP ammonium salt (21 Ci mmol^{-1}, Amersham) for binding to cyclic AMP-dependent protein kinase from bovine adrenal cortex (BDH). Recovery of cyclic AMP (60 pmol ml^{-1}) added to the washed platelet suspension, extracted and assayed as above was 85 ± 5% (n = 5). Also recovery of radioactivity following addition of labelled cyclic AMP to the washed platelet suspension, extraction and dissolution in buffer was 94 ± 3% (n = 5). Log concentration-inhibition curves were plotted and IC_{50} values calculated; for fresh platelets these corresponded to 55% of control cyclic AMP (taking maximum inhibition as 10% of control) and for stored platelets to 60% of control (maximum = 20%).

Compounds

11-Deoxy PGE_2-1-alcohol (starting material *nat* PGA_2) and EP 092 (*rac* 9α,11α-ethano-ω-heptanor-13-methyl-13-phenylthiocarbamoylhydrazino-prosta-5Z-enoic acid) were prepared in our laboratory. The following compounds were gifts: sulprostone, PGI_2 sodium salt and cicaprost from Prof. H. Vorbruggen, Schering AG, Berlin, Germany; MB 28767 (*rac* 15S- hydroxy-9-oxo-16-phenoxy-ω -tetranor-prost- 13E- enoic acid) from Dr M. Caton, Rhone-Poulenc, U.K.; misoprostol from Dr P. Collins, G.D. Searle, U.S.A.; butaprost from Dr P. Gardiner, Bayer, U.K.; AH 6809 (6-isopropoxy-9-oxoxanthene-2-carboxylic acid), GR 32191 (9α-(biphenylyl)methoxy-11β- hydroxy-12β -(N-piperidinyl)-ω -octanor- prost-4Z -enoic acid) and GR 63799X (13-oxa-13,14-dihydro-16-phenoxy-ω-tetranor PGE_2-4-(benzoylamino)phenyl ester) from Dr R.A. Coleman, Glaxo, U.K. PGE_2, 16,16-dimethyl PGE_2, 17-phenyl-ω-trinor PGE_2, 11-deoxy PGE_1 and U-46619 (11,9-epoxymethano PGH_2) were purchased from Cayman Chemicals, U.S.A. Ethanolic stock solutions of the prostanoids (10–30 mM) were stored at −20°C and diluted with 0.9% NaCl solution (saline) for use. Due to its low water solubility, GR 63779X was stored in ethanol at a concentration of 0.4 mM. Saline dilutions of 20 μM and less were prepared and used for one day only. An aliquot of a 2 mg ml^{-1} chloroform solution of PAF (L-isomer, Sigma) was evaporated to dryness with a nitrogen jet and the residue dissolved in saline to give a 10^{-4} M stock solution. A 10^{-3} M ADP stock solution was prepared by dissolving ADP (Grade III, Sigma) in saline and adjusting the pH to 6.8 by addition of solid $NaHCO_3$.

Results

Potentiation of aggregation in human PRP

Initial observations on sulprostone and PGE_2 Shape change and aggregation in citrated human PRP were recorded by the conventional light scattering method. In about half of the 31 donors examined, sulprostone between 4 and 400 nM had no effect on the oscillatory signal associated with stirring a suspension of non-activated (discoid) platelets. In the remaining donors, 4–50 nM sulprostone also had no effect, but at 100–400 nM a slow increase in light transmission with some decrease in oscillation was seen (see Figure 1a, sulprostone 100 nM). This type of response was quite different from the rapid shape change (decrease in light transmission with loss

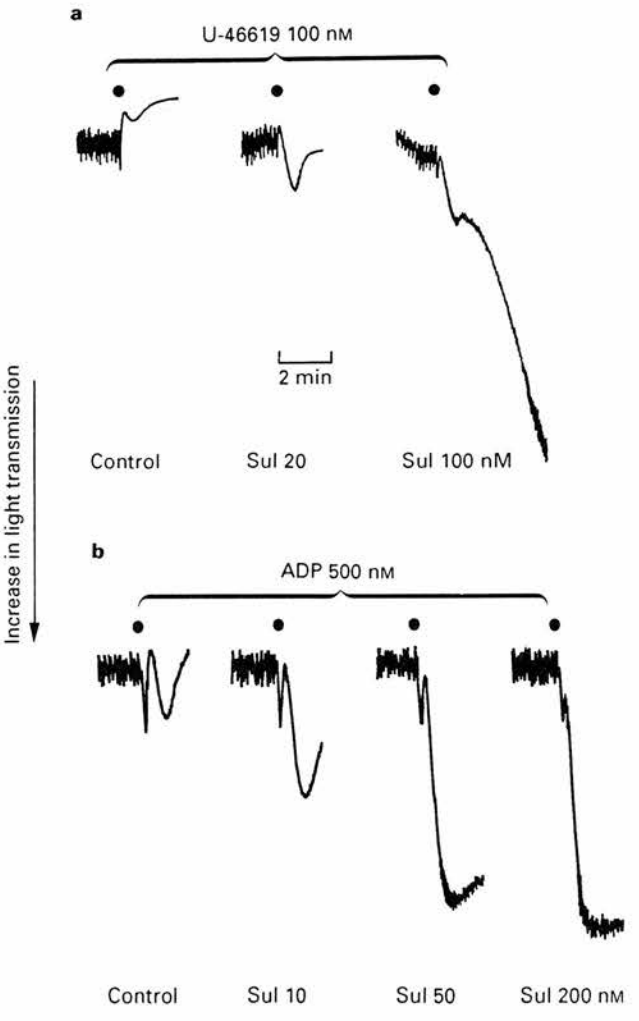


Figure 1 Light transmission records showing the potentiating effect of sulprostone (Sul) on platelet aggregation in human PRP induced by (a) U-46619 (no antagonists present) and (b) ADP in the presence of 1 μM EP 092. U-46619/ADP were added at (●) and sulprostone was added 2 min before each dose of aggregating agent.

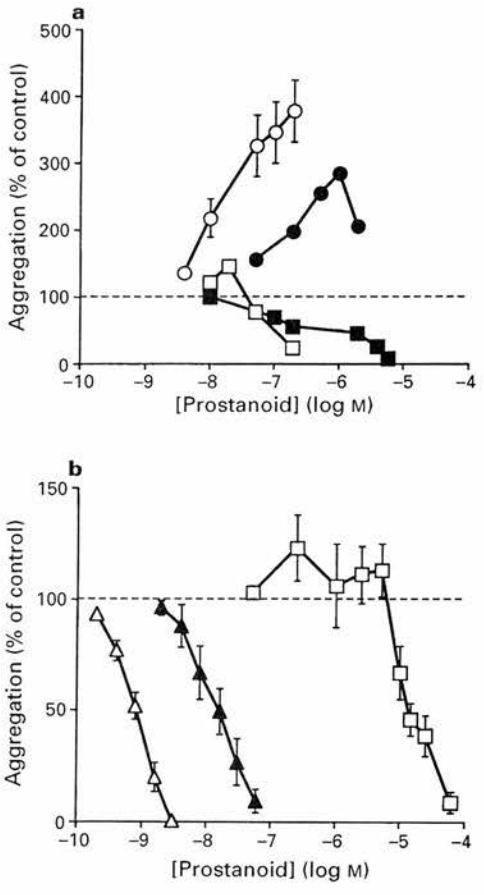


Figure 2 Log concentration-response curves for platelet aggregation in human platelet-rich plasma (PRP) in the presence of GR 32191 (0.5 μM). Control response = 100%. Vertical bars indicate s.e.mean. (a) Effects of sulprostone (○) (mean of 8 donors) and prostaglandin E_2 (PGE_2, ●, □, ■) (three individual donors) on small primary waves induced by ADP. (b) Effects of cicaprost (△) ($n = 8$), PGE_1 (▲) ($n = 4$) and PGE_2 (□) ($n = 4$) on 75% maximal aggregation responses induced by ADP.

of oscillation) and reversible aggregation induced by low concentrations of ADP (500 nM), PAF (20 nM) or U-46619 (100 nM) (Figure 1). 'Slow waves' to sulprostone were unaffected by the TP-receptor antagonists EP 092 (1 μM) and GR 32919 (0.5 μM); both antagonists blocked shape change/reversible aggregation responses to U-46619, but not those to ADP and PAF.

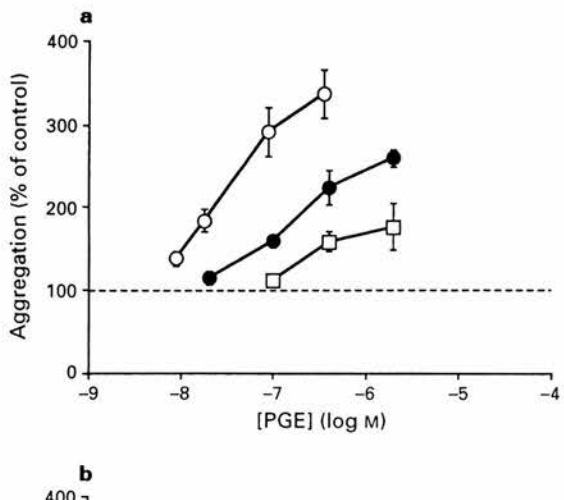
Irrespective of whether slow waves were seen, sulprostone (4–400 nM) consistently potentiated aggregation responses to ADP, PAF and U-46619. At the lower sulprostone concentrations the small reversible aggregation wave to a fixed concentration of each aggregating agent was increased in size. At the higher concentrations the potentiation was great enough to elicit secondary aggregation, particulary with U-46619 (Figure 1a). Although EP 092 (1 μM) and GR 32191 (0.5 μM) abolished secondary aggregation, they did not inhibit sulprostone potentiation of primary aggregation waves to ADP (Figure 1b) and PAF. The log concentration-response curve (mean values using PRPs from 8 donors) for sulprostone potentiation of ADP-induced aggregation in the presence of 0.5 μM GR 32191 is shown in Figure 2a.

Unlike sulprostone, PGE_2 showed variable effects on small primary waves induced by ADP. In PRPs from 2 of the 8 donors investigated PGE_2 (10–5000 nM) only inhibited aggregation, the log concentration-response curves being rather shallow (Figure 2a, filled squares). In the remaining 6 PRP samples, potentiation of aggregation was seen at the lower concentrations of PGE_2 tested (10–50 nM). As the concentration of PGE_2 was increased, either a bell-shaped potentiation curve was obtained (Figure 2a, filled circles) or a switch to inhibition of aggregation was observed (Figure 2a, unfilled squares).

For comparison, the effects of cicaprost (a stable prostacyclin analogue), PGE_1 and PGE_2 on 75% maximal aggregation responses to ADP in the presence of GR 32191 (0.5 μM) are shown in Figure 2b. Cicaprost was a highly potent inhibitor of aggregation ($IC_{50} \sim 0.8$ nM). PGE_1 also inhibited aggregation, being 15–20 times less potent than cicaprost and having a slightly shallower log concentration-response curve. PGE_2 (50–5000 nM) potentiated aggregation such that maximum aggregation was often produced; between 10 and 62.5 μM PGE_2 always inhibited aggregation.

Effect of AH 6809 In three experiments, the EP_1-receptor antagonist, AH 6809, at 10 μM had no effect on sulprostone potentiation of ADP-induced aggregation. With 100 μM AH 6809, there was a modest parallel shift to the right of the sulprostone log concentration-response curve, giving dose-ratios of 2.0, 3.2 and 4.4.

Activities of other PGE analogues Preliminary experiments appeared to show that the potentiating effect of PGE_2 was favoured over its inhibitory effect when PAF was used as the aggregating agent in place of ADP. The effects of nine PGE



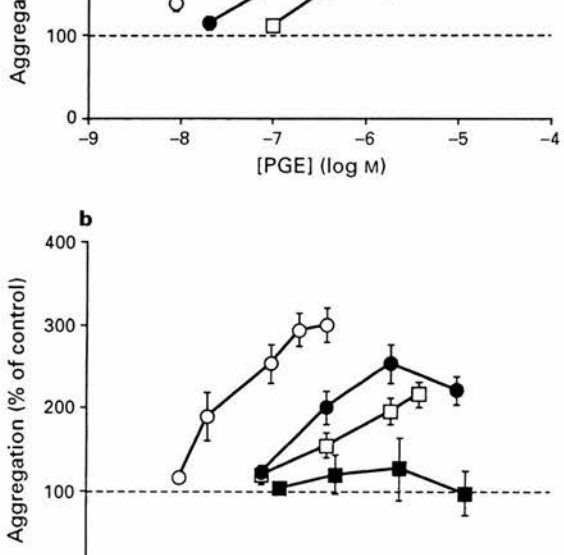


Figure 3 Effects of prostaglandin E (PGE) analogues on PAF-induced aggregation in human platelet-rich plasma (PRP) in the presence of GR 32191 (0.5 μM) and BW A868C (0.2 μM). Control response = 100%. Vertical bars indicate s.e.mean. (a) Sulprostone (○) ($n = 6$); MB 28767 (●) ($n = 4$); GR 63779X (□), ($n = 4$). (b) 16,16-dimethyl PGE_2 (○); misoprostol (●); 17-phenyl-ω-trinor PGE_2 (□); 11-deoxy PGE_2-1-alcohol (■) (all $n = 4$).

analogues were therefore tested for their ability to potentiate PAF-induced aggregation in PRP. In addition to GR 32191 (0.5 μM), BW A868C (0.2 μM) was added to prevent any inhibition of aggregation through activation of DP-receptors. GR 32191 was preferred to EP 092 because the block of TP-receptors produced by the former is more difficult to surmount (see Tymkewycz *et al.*, 1990). Six of the analogues, sulprostone, 16,16-dimethyl PGE_2, MB 28767, misoprostol, GR 63779X and 17-phenyl-ω-trinor PGE_2 showed consistent potentiating activity (Figure 3), although the log concentration-response curves for misoprostol, MB 28767 and in particular GR 63779X and 17-phenyl-ω-trinor PGE_2 were shallower than those of sulprostone and 16,16-dimethyl PGE_2. Equi-effective molar ratios (EMR) calculated at the 150% control level are given in Table 1. The testing of GR 63779X was affected by the high proportion of ethanol required to solubilize this prostanoid. Thus 20 mM ethanol (corresponding to 0.4 μM GR 63779X) did not affect PAF aggregation; 100 mM ethanol (2 μM GR 63779X) reduced the PAF response by 11 ± 3% ($n = 4$) and 200 mM ethanol by 51 ± 6%. At the highest concentrations of misoprostol and 11-deoxy PGE_2-1-alcohol used, the ethanol concentration in the PRP was 20 mM.

PGE_2 (20–1000 nM) and 11-deoxy PGE_2-1-alcohol (100–10,000 nM) still showed variable actions. In some donors, potentiation with a shallow log concentration-response curve was seen and in others there was evidence of inhibition of the PAF aggregation. In the final analysis it appears that the use of PAF may not have a significant advantage over ADP. Butaprost (10–3000 nM) inhibited only PAF aggregation ($IC_{50} = 0.95$, 1.3, 1.5 μM, 3 donors).

Inhibition of cyclic AMP production

Due to difficulties in obtaining large quantities of fresh human blood, some experiments were performed on time-expired platelet concentrates supplied by the local blood transfusion service. Cyclic AMP levels in suspensions of the washed platelets were measured with a radiolabelled cyclic AMP/protein binding assay. Elevations of cyclic AMP induced by cicaprost in the presence of 0.5 μM GR 32191 are shown in Figure 4a; Fresh and stored platelets gave similar log concentration-response curves; EC_{50} values were about 10 and 8.5 nM respectively. For all subsequent inhibition experiments, a cicaprost concentration of 8 nM was chosen to induce a submaximal (control) rise in cyclic AMP.

The effects of pre-incubation of the PGE analogues on the cicaprost-induced rise in cyclic AMP in the presence of 0.5 μM GR 32191 are shown in Figure 4. All of the PGE analogues inhibited cyclic AMP accumulation with the exception of butaprost; IC_{50} values and EMR are given in Table 1. The slopes of the inhibition curves for the stored platelets were somewhat shallower than those of the fresh platelets, although sensitivities to sulprostone were similar ($IC_{50} = 2.2$ and 2.0 nM respectively).

AH 6809 at a concentration of 10 μM shifted the log concentration-response curves to sulprostone and 17-phenyl-ω-trinor PGE_2 to the right (dose-ratio = 7.2 and 3.9 respectively) (Figure 4b). pA_2 values calculated using the Schild equation are 5.75 and 5.32 respectively.

Table 1 Agonist potencies of prostaglandin E (PGE) analogues relative to sulprostone

	Equi-effective molar ratio			
PGE analogue	*Potentiation of PAF aggregation in human PRP*	*Inhibition of cyclic AMP in human washed platelets*	*Contraction of guinea-pig trachea*	*Inhibition of guinea-pig vas deferens*
Sulprostone	1.0 (EC_{150} = 10 nM)	1.0 (IC_{50} = 2.2/2.0 nM*)	1.0 (EC_{50} = 7 nM)	1.0 (IC_{50} = 0.2 nM)
16,16-Dimethyl PGE_2	1.3	0.34*	0.022	0.93
MB28767	6.8	2.1*	>150	5.0
PGE_2	↓	2.5	↓	7.1
Misoprostol	13	10.3*	↓	3.6
GR 63779X	26	20	14	1.7
17-Phenyl-ω-trinor PGE_2	29	37	0.32	45
11-Deoxy PGE_2-1-alcohol	↓	180	↓	79
Butaprost	↓	>350	↓	>7000

Human platelet data were obtained in the present study. Guinea-pig trachea and vas deferens values are from Lawrence *et al.*, 1992, except those for GR 63779X which are our unpublished values, in agreement with Bunce *et al.*, 1990. *Values for stored platelets. ↓ Inhibitory effect observed – assessment of stimulant potency not possible.

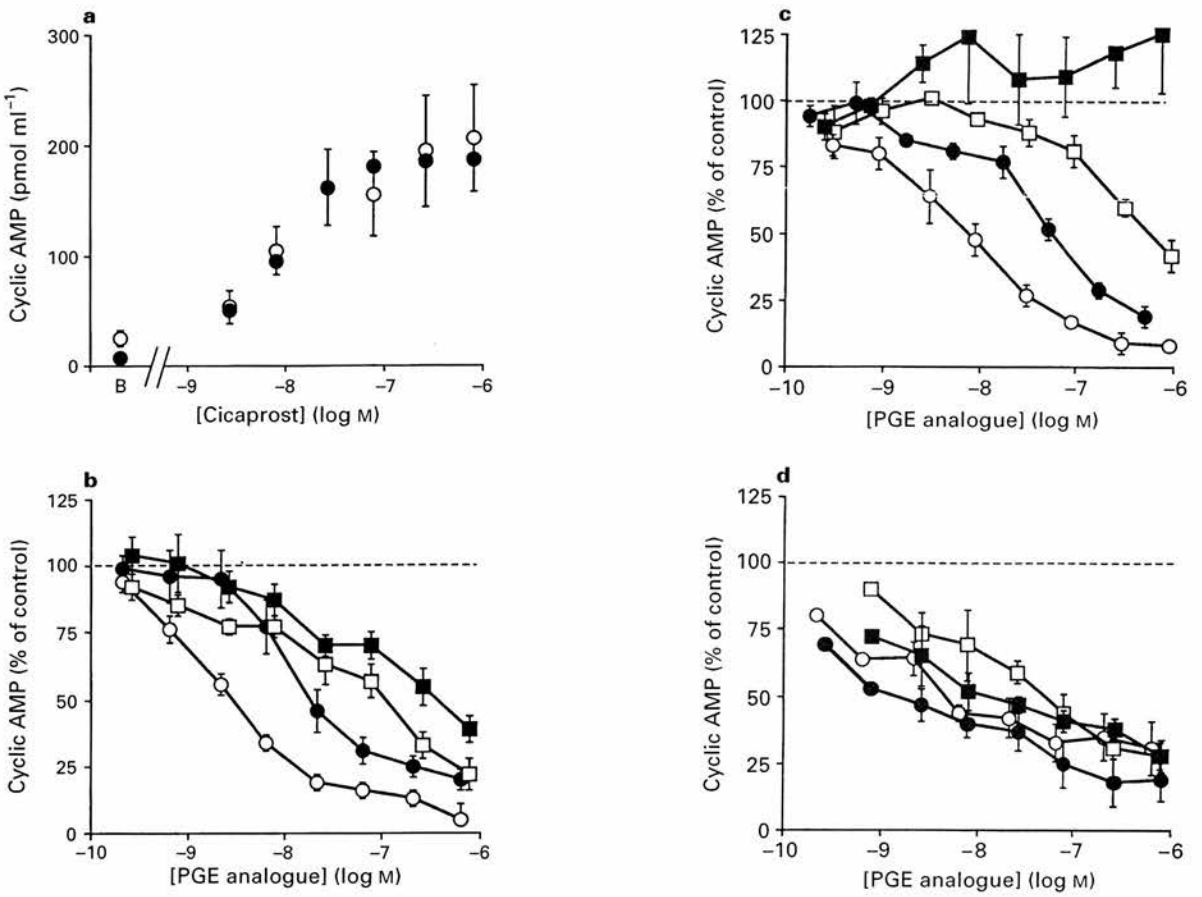


Figure 4 Effects of prostaglandin E (PGE) analogues on cicaprost-induced elevation of cyclic AMP in human washed platelets. GR 32191 (0.5 μM) was present in all experiments. Vertical bars indicate s.e.mean; $n = 4$ in (b) (c) and (d). (a) Elevation of cyclic AMP by cicaprost in fresh (○) ($n = 6$) and stored (●) ($n = 3$, no error bars) platelets. B = basal condition; 8 nM cicaprost was used in (b) (c) and (d); cyclic AMP generated = 100%. (b) Fresh platelets: inhibition curves for sulprostone (○); sulprostone in the presence of 10 μM AH 6809 (●); 17-phenyl-ω-trinor PGE_2 (□) and 17-phenyl-ω-trinor PGE_2 in the presence of 10 μM AH 6809 (■). (c) Fresh platelets: inhibition curves for PGE_2 (○); GR 63779X (●); 11-deoxy PGE_2-1-alcohol (□) and butaprost (■). (d) Stored platelets: inhibition curves for sulprostone (○); 16,16-dimethyl PGE_2 (●); misoprostol (□) and MB 28767 (■).

Discussion

We have shown that several analogues of PGE_2 consistently potentiate ADP- or PAF-induced aggregation in human PRP in the presence of a specific TP-receptor antagonist such as EP 092 (Armstrong *et al.*, 1985) or GR 32191 (Lumley *et al.*, 1989). Sulprostone and 16,16-dimethyl PGE_2 were the most active agents, with threshold responses seen at about 2 nM in some PRPs. However sulprostone was chosen as the standard agonist since it is a weaker TP-receptor agonist than 16,16-dimethyl PGE_2. For example, 16,16-dimethyl PGE_2 can elicit irreversible aggregation in human PRP and is a medium potency full agonist on the rat aorta, rabbit aorta and dog saphenous vein (see Tymkewycz *et al.*, 1991), whereas sulprostone does not induce typical thromboxane-like aggregation and is a very weak agonist on the rat aorta (Coleman *et al.*, 1988) and rabbit aorta (R.L. Jones, unpublished observations).

Two factors may affect estimations of the relative potentiating potencies of the PGE analogues under investigation. First, there may be differences in the amounts of the PGE analogues bound to plasma protein in the PRP. Secondly, and probably of greater importance, the human platelet contains a highly sensitive IP-receptor-adenylate cyclase system which when activated inhibits aggregation. This is illustrated in our experiments by the high potency of cicaprost (IC_{50} against ADP-induced aggregation = 0.8 nM). Thus the mixed effects of PGE_2 on PAF aggregation in the presence of the potent and specific DP-receptor antagonist BW A868C (Giles *et al.*, 1989) are most easily explained by a concurrent activation of EP- and IP-receptors over the 50 nM to 5 μM concentration-range. Previous studies with the weak DP-receptor antagonist, N-0164, have produced conflicting results for PGE_2. In the earlier study, N-0164 suppressed the platelet inhibitory action of PGD_2 and PGE_2, but did not affect the inhibitory actions of PGE_1 (MacIntyre & Gordon, 1977), whereas in a later study the inhibitory actions of PGI_2, PGE_1 and PGE_2 were unaffected (Tynan *et al.*, 1984). The shallower log concentration-potentiation curves for MB 28767, misoprostol, 17-phenyl-ω-trinor PGE_2 and 11-deoxy PGE_2 1-alcohol could also reflect IP-receptor activation by these analogues at concentrations of 1 μM and above.

Taking the EC_{150} as a measure of potentiating potency, then the ranking is as follows: sulprostone = 16,16-dimethyl PGE_2 > MB 28767 ≥ misoprostol > GR 63779X = 17-phenyl-ω-trinor PGE_2. The high potency of sulprostone as a potentiator implies that an EP_2-receptor is unlikely to be involved. EP_2-receptors are associated with the relaxant actions of PGE_2 on vascular and respiratory smooth muscle and sulprostone is virtually inactive in these systems (Coleman *et al.*, 1987a,b; Lawrence & Jones, 1992). Furthermore it is likely that the EP_3 rather than the EP_1 subtype mediates the potentiating effect, since sulprostone and MB 28767 are more potent than 17-phenyl-ω-trinor PGE_2 on the guinea-pig vas deferens (EP_3 preparation) and *vice versa* for the guinea-pig trachea (EP_1 preparation) (Lawrence *et al.*, 1992) (see Table 1). It should be noted that we are using the guinea-pig vas deferens as the standard EP_3 preparation, since there is

now evidence (Lawrence & Jones, 1992) that the chick ileum, the original EP_3 preparation (Coleman *et al.*, 1987a,b), contains more than one EP-receptor.

PGE_1 is a highly potent EP_3 agonist, being slightly more potent than PGE_2 and about 5 times less active than sulprostone (see Coleman *et al.*, 1990). But for its prostacyclin-like activity we would expect it to potentiate aggregation at concentrations of 30 nM and above. The slightly shallower inhibition curve for PGE_1, also observed previously by Andersen and colleagues (1980), may reflect the existence of an opposing potentiation.

Preliminary experiments on human washed platelets showed that sulprostone produced marked potentiation of aggregation, but mixed effects were still seen with PGE_2 and 11-deoxy PGE_2-1-alcohol. Because of this and the greater difficulties of working with washed platelets, we felt that it was hardly worthwhile attempting to obtain potentiating potencies on washed platelets. Instead we concentrated our efforts on developing an assay based on the putative second messenger mechanism, namely inhibition of adenylate cyclase in a low protein system. Our aim was to accentuate the EP agonist action of a PGE analogue at the expense of its IP agonist action. Cicaprost was chosen as the agent to raise the cyclic AMP level since it is a stable and specific IP-receptor agonist. In particular it lacks the potent EP_1-receptor agonist activity shown by other carbacyclins, such as iloprost and 6a-carba PGI_1 (Dong *et al.*, 1986; Lawrence *et al.*, 1992). Furthermore it has less EP_3-agonist activity than iloprost and carbacyclin on the guinea-pig vas deferens (Lawrence *et al.*, 1992). Ashby (1992) has suggested from analysis of the time course of cyclic AMP accumulation in intact human platelets that both iloprost and cicaprost activate an inhibitory receptor to reduce cyclic AMP accumulation. However the initial rise in cyclic AMP is only rapidly suppressed at high concentrations (3 μM) of each prostacyclin analogue and it is easily possible that the slow fade of the cyclic AMP level seen with lower concentrations (30 nM) is due to a mechanism other than activation of an inhibitory receptor. In our experiments we deliberately used a low concentration of cicaprost (8 nM, $\sim EC_{50}$, Figure 4a), which is unlikely to activate the EP-receptor. In addition submaximal stimulation of adenylate cyclase should allow the greatest opportunity for PGE-induced inhibition of the enzyme complex. The success of our strategy can be judged by the high sensitivities and monophasic inhibition curves obtained for both sulprostone and PGE_2 ($IC_{50} = 2.2$ and 5.4 nM respectively).

Reconsidering the nature of the EP-receptor involved, the low potency of 17-phenyl-ω-trinor PGE_2 relative to sulprostone and MB 28767 again indicates that the EP_1-receptor subtype is unlikely to be involved. Furthermore AH 6809 shows a much weaker blocking effect ($pA_2 = 5.3/5.76$) than would be expected for EP_1-receptor antagonism (pA_2 on guinea-pig trachea = 7.35, 17-phenyl-ω-trinor PGE_2 as agonist, Lawrence *et al.*, 1992). The even lower pA_2 value ($\sim$4.3) obtained for block of sulprostone potentation by AH 6809 in the aggregation assay is probably due to plasma protein binding of the antagonist. Coleman and colleagues (1985) have shown that about 98% of AH 6809 is bound to 4% defatted bovine serum albumen. In our PRP system, 90–95% binding of AH 6809 to the 2% total plasma protein present would account for the difference between the pA_2 values. AH 6809 is not a particularly specific EP_1 antagonist and will also block DP- and TP-receptors on human washed platelets at a concentration of 10 μM ($pA_2 = 6.3$ and 5.9, calculated from data in Keery & Lumley, 1988).

Finally, how good is the correlation between the potencies of the PGE analogues for inhibition of adenylate cyclase in human platelets and for inhibition of the twitch response of the guinea-pig vas deferens, the standard EP_3 preparation (Table 1)? Using log EMRs, the least squares regression line has a slope of 1.00 and a correlation coefficient of 0.80 ($P < 0.05$). However, this is not a particularly convincing correlation. The greatest discrepancy is the higher potency of GR 63779X on the vas deferens compared to the adenylate cyclase system (and also the aggregation assay). GR 63779X has an agonist specificity similar to sulprostone ($EP_3 > EP_1 >> EP_2$) (Bunce *et al.*, 1990) and although there are clearly difficulties in its use owing to its low water solubility, we feel that our potency estimations are valid. Consequently we consider it prudent to err on the side of caution and pending further studies to refer to the EP-receptor in the human platelet as 'EP_3-like'.

It seems reasonable to propose from our experiments and the studies of Ashby (1988) that activation of EP_3-like receptors on the platelet membrane inhibits adenylate cyclase through the intermediary of G_i, and this leads to potentiation of aggregation. Other human platelet receptors known to operate in a similar manner are the ADP receptor (a type of P_2-purinoceptor) and the α_2-adrenoceptor (see Hourani & Cusack, 1991, for a review of this area). In an elegant study Brass and colleagues (1988) have investigated the nature of the G-proteins mediating pertussis toxin-sensitive phospholipase C activation (G_p) and adenylate cyclase inhibition (G_i) in human platelets. Thrombin stimulated PI hydrolysis and inhibited cyclic AMP accumulation and this was associated with >90% ADP-ribosylation of the extracted G-protein. In contrast, adrenaline only inhibited cyclic AMP accumulation in concert with 50% ADP-ribosylation; U-46619 neither inhibited cyclic AMP nor induced pertussis toxin-sensitive PI hydrolysis. It appears that the G-proteins regulating both PI hydrolysis and inhibition of cyclic AMP formation in this system are very similar in nature and the possibility that they are the same chemical species has been raised. Clearly it would be of interest to investigate by similar methods the nature of the G-protein(s) interacting with the EP_3-like receptor of the human platelet; from our studies sulprostone would be the most useful agonist for this purpose.

In conclusion, our results are relevant to the therapeutic use of PGE analogues in man. TP-agonist activity in a PGE analogue is an obvious risk factor. However, the possibility of a PGE analogue potentiating platelet aggregation via EP_3-like receptors should also be taken into consideration.

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